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13. ABSTRACT ***(Maximum 200 Words)***

The objectives of this study are 1) to identify factors that regulate the growth and differentiation of organoids formed by two types of normal human breast epithelial cells (HBEC) in Matrigel; 2) to characterize the expression and function of estrogen receptors (ER) in normal and *in vitro* neoplastically transformed HBEC; and 3) to determine if a HBEC type with stem cell characteristics (Type I) is more susceptible to telomerase activation and immortalization. The major results are 1) Type I HBEC in conjunction with Type II cells are capable of forming ductal and end bud or lobule 1-like structures in Matrigel which preserve the undifferentiated state of HBEC for a long time, evidence that Type I HBEC are stem cells; 2) Type I normal HBEC and their neoplastically transformed clones express a variant ER *in vitro* on plastic while expressing a wild type ER in tumors developed in nude mice or grown *in vitro* in Matrigel; 3) high susceptibility of Type I HBEC to telomerase activation and immortalization; and 4) the lifespan of HBEC can be effectively extended by co-transfection with a dominant-negative mutant p53 and the human c-myc. These findings indicate Type I stem cells as targets for carcinogenesis and inactivation of p53 and activation of telomerase as major events in initial stage of breast carcinogenesis.

14. SUBJECT TERMS
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V. INTRODUCTION

We have previously developed a culture method to grow two morphologically distinguishable types of normal human breast epithelial cells (HBEC) from reduction mammoplasty (1). These two types of cells are substantially different in many phenotypes (1-3) as listed in Table 1. The most significant characteristics of Type I HBEC related to breast carcinogenesis are (a) the expression of estrogen receptors (ER); (b) stem cell features (i.e., ability of Type I cells to differentiate into Type II cells and to form budding/ductal structures on Matrigel); and (c) the high susceptibility to neoplastic transformation by oncogenic stimulus (i.e., SV40 large T-antigen induced anchorage independent growth and high frequency of immortalization) (1, 4). Since breast cancers are very likely to be derived from stem cells and ER-positive HBEC, the major objectives of this project are (a) to develop and characterize an *in vitro* organoid system using Type I and Type II HBEC for analyzing factors that regulate normal and abnormal growth and differentiation of human mammary gland and for studying the mechanism of carcinogenesis; and (b) to characterize the structure/function and regulation of estrogen receptors expressed in normal and *in vitro* neoplastically transformed cell lines.

The mechanism that Type I HBEC were more susceptible to neoplastic transformation is not known. In general, tumor cells are known to express telomerase that maintains telomere length for continuous growth whereas

normal cells lack the telomerase activity (5). One study, however, concludes that telomerase activity is a biomarker of cell proliferation but not malignant transformation in human cells including breast epithelial cells (6). Since we have the putative human breast epithelial stem cells and have developed and preserved a series of SV40 transformed Type I and Type II HBEC at different passages, we are in a good position to determine whether human breast epithelial stem cells express telomerase and whether the innate level of telomerase activity in Type I HBEC is sufficient for immortalization or readily activated during neoplastic transformation. This became an additional task for this project (Task 9).

One of the task (Task 5) proposed to study the expression of ER in ionizing radiation transformed HBEC. Although we have shown that ionizing radiation is capable of extending the lifespan of Type II HBEC (7) and converting a SV40 immortalized Type I HBEC line into tumorigenic cells (8), we were unsuccessful in immortalizing Type I cells by x-rays. Therefore, we decided to use co-transfection with a dominant-negative p53 and a human c-myc. To our surprise, the technique appears to be very effective in extending the lifespan of HBEC. Most importantly, the resulting transformed clones expressed the ER and grew in soft agar. This represents a new approach to this task.

VI. BODY

A. Characterization of Budding and Ductal Structures Formed by Normal HBEC in Matrigel (Task 1)

The results of this study was reported in great detail in the second year Annual Report (1998) in regard to 1) conditions to form budding/ductal structures; 2) the types of organoids formed by the two types of HBEC; and 3) the alternate growth of HBEC as monolayer on plastic and organoid in Matrigel to preserve the undifferentiated state of HBEC for extended time. This project is largely completed except for the immunofluorescent staining of the organoids to reveal gene expression. The techniques to section the organoid developed in Matrigel and to reveal gene expression by immunostaining have been developed. Using this technique, we found that the centers of the spherical balls formed by Type II HBEC in Matrigel are not hollow as they appear. The structure (Fig. 1) is actually similar to the squamous metaplasia developed by rat mammary organoid in Matrigel (9). The Type II HBEC expressed cytokeratin 14 when grown on plastic as monolayer (1). In spherical structure, only the outermost cell layer that contacted with Matrigel showed the expression of cytokeratin 14 (Fig. 2) similar to basal epithelial cells in human mammary gland. The technique will be applied to budding/ductal structures formed by Type I and Type II HBEC to reveal their structure and gene expression (i.e., ER and cytokeratins) by immunostaining. Part of the results are included in a paper published in

Cancer Research (4) and *Radiation Research* (10). A manuscript to report the bulk of the study is in preparation.

B. The Effects of Hormones and Growth Factors on Growth and Differentiation of Budding/Ductal Structures Formed by Normal HBEC on Matrigel (Task 2)

The budding/ductal organoids can be formed in Matrigel in our defined MSU-1 Medium supplemented with EGF, insulin, hydrocortisone, estradiol and human transferrin. The former three are essential for the growth of the two types of HBEC as monolayer on plastic (2). In our preliminary experiments, we also found that EGF, insulin and hydrocortisone are essential for the development of budding/ductal structure formed by the two types of HBEC on Matrigel. Fetal bovine serum which inhibits the growth of Type II cells but not Type I cells was found to inhibit the growth of budding/ductal organoids. In the past years, we found that the formation and development of organoids can be influenced by different cell cultures derived from different women and by the use of different lots of commercial Matrigel. We are experimenting on these parameters to obtain ideal organoids under optimum conditions. Then we will test the effects of individual and combination of growth factors and hormones.

C. Comparison of Estrogen Receptor (ER) Expression in Two Types of Normal HBEC Grown on Plastic and in Matrigel (Task 3)

A major difference between Type I and Type II HBEC is the expression of ER. In contrast to Type II cells, Type I HBEC were ER-positive as shown by immunostaining, RT-PCR and Western blotting (3). The latter showed that the ER expressed is a 48 kd variant ER. The *in vitro* neoplastically transformed Type I cells also expressed the 48 kd ER when grown on plastic *in vitro*. The same cells, however, expressed the 66 kd wild type ER and a 51 kd variant ER *in vivo* as tumors developed in immune-deficient mice. These results were included in the first year annual report (1997). In addition, we reported that Matrigel was able to mimic the *in vivo* condition in inducing the expression of the wild type ER (66 kd), indicating a role of extracellular matrix in the regulation of ER expression. We also demonstrated that the structure of the 51 kd variant ER expressed *in vivo* (tumor in nude mice) and in Matrigel culture is clearly different from the 48 kd variant ER expressed in cells grown on plastic, i.e., an anti-ER antibody recognizing the N-terminal portion of ER can detect both the wild type ER and the 51 kd ER but not the 48 kd ER (Fig. 18 in 1998 Annual Report).

The major results of this study have been published (3) and presented at the Annual Meeting of the American Association for Cancer Research (Sun, W. and C.C. Chang, 1998. Matrigel mimics the *in vivo*

condition in activating the expression of the wild type estrogen receptor in a human breast epithelial cell type. *Proc. Am. Assoc. Cancer Res.* 39:407). In the past year, we further defined the molecular structure and promoter usage of the variant ER by RT-PCR analysis as described in the following.

RT-PCR Analysis of the Structure of a Variant ER, and Promoter Usage in Type I HBEC.

In our previous study using a primer pair encompassing exon 2 in RT-PCR analysis, we found no ER-α transcript in Type I HBEC and concluded that the variant ER-α expressed in Type I HBEC has deleted the exon 2 (3). The primer pair is located near the translation starting site in exon 1 and in exon 3. Using a different primer pair (E1U new near the 3′ end of exon 1 and E5L in exon 5, we found the 850 bp transcript was expressed in a transformed Type I cell line (M13SV1R2-2) grown on plastic and the MCF-7 breast cancer cell line which expressed the wild type ER-α (Fig. 3). Therefore, exons 2, 3 and 4 are present in the 46 kd variant ER and the deletion in the variant ER is upstream of the primer E1U new site in exon 1. Indeed, from the molecular weight calculation, the deletion of exon 2 alone will produce a 59 kd ER whereas the deletion of exon 1 will result in a 49 kd protein which is close to the observed 46-48 kd ER-α in Type I HBEC. The translation of the variant ER-α at the 3′ end of exon 1 is possible since there is a translation starting codon (ATG) at 557-559 bp (the upstream ATG at 233-235 bp).

Three promoters have been identified for the transcription of human estrogene-α gene (20). Using the same primer pairs for RT-PCR analysis of the usage of promoter A, B and C, we found that Type I normal HBEC used promoter A but not promoter C. However, the immortal (M13SV1) or tumorigenic cell line (M13SV1R2N1) derived from Type I HBEC used both promoter A and C similar to MCF-7 (Fig. 4). A low level of promoter B usage was found for both Type I and Type II HBEC as well as the immortal (M13SV1) and tumorigenic (M13SV1R2N1) Type I HBEC, similar to the ER-positive breast cancer cell lines MCF-7 and T47D. The ER-negative cancer cell line MDA-MB-231 did not use any of the 3 promoters.

D. Estrogen Receptor Expression in SV40 Large T-antigen Transformed Human Breast Epithelial Cells (Task 4)

This task has been completed. A detailed report was presented in the 1997 Annual Report. A paper was published on this work (Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang, 1997. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* 18:251-257).

E. Estrogen Receptor Expression in Ionizing Radiation Transformed HBEC (Task 5)

We have characterized a series of immortal, weakly tumorigenic and highly tumorigenic cell lines derived from Type I HBEC following sequential treatment with SV40 large T-antigen, x-rays and neu oncogene (Kang, K.S. et al. 1998. Involvement of tyrosine phosphorylation of $p185^{C\text{-}erbB2/neu}$ in tumorigenicity induced by x-rays and neu oncogene in human breast epithelial cells. *Molecular Carcinogenesis* 21:225-233. Reprint submitted in 1998 Annual Report). This paper (8) is one of the few reported successes in the creation of human tumour cells *in vitro* as cited by a *Nature* paper (11) by Dr. Robert A. Weinberg. These immortal or tumorigenic cells, when grown on plastic, expressed the 48 kd variant ER (3). The tumorigenic cell lines expressed the wild type ER *in vivo* or in Matrigel as described in the previous section.

The original proposal plans to characterize the expression of ER in HBEC immortalized by x-rays. These immortal cells are to be produced by a different project supported by a different grant from NIEHS. Now since that project only produced extended life clones (7) but not immortalized HBEC, we decided to immortalize our cells by co-transfection with a dominant-negative mutant p53 and a human c-myc.

A detailed report of the co-transfection of Type II HBEC with a dominant-negative mutant p53 and the human c-myc plasmids was

provided in last year's annual report. The study showed that the co-transfection was very effective in extending the lifespan of Type II HBEC but was unsuccessful for Type I HBEC. A total of 11 clones was obtained from two experiments. Their lifespan ranges from 36 to 95 cumulative population doubling level (cpdl). Interestingly, these clones showed mixed phenotypes of Type I and Type II cells, i.e. ability to grow in soft agar (AIG^+) (4/4), expression of estrogen receptor in Western blotting (6/7) and competence in gap junctional intercellular communication (4/5). At early passages (20-30 cpdl), the telomerase activities of 8 clones examined have not been activated. Therefore, we attempted to transfect one of the cell lines (M21-32) with the hTERT plasmid obtained from Dr. Robert Weinberg. Several transfectant clones isolated by selection with hygromycin, however, did not show an elevated level of telomerase activity by the TRAP assay. Recently, we found that the M21-32 cell line without hTERT transfection was able to continue to grow after 95 cpdl. These cells may have been immortalized. This will be verified by the assay of its telomerase activity soon. The updated phenotypes of these clones are listed in Table 2.

In different series of experiments, we attempted to extend the lifespan or to immortalize HBEC by transfection with cyclin D1 or the catalytic subunit of human telomerase gene, hTERT. Both were not successful.

For Cyclin D1 experiments, we used the pcDNA3-cyclin D1 plasmid with G418 resistance selective marker (provided by Dr. Y.H. Choi). Both Type I and Type II HBEC from HME30 (approximately 6 x 10^6 and 1 x 10^6 respectively) were transfected by the plasmids mediated by lipofectin (Life Technologies). After selection with G418, only 3 actively proliferating G418-resistant colonies from Type II cells were found. These cells were able to grow initially after transferred to 25 cm^2 flasks but later failed to show sustained growth. They are not considered as clones with extended lifespan using SV40 transformed clones as a criterion (>25 cpdl).

Ectopic expression of hTERT was previously shown to be sufficient to immortalize HBEC with inactive $p16^{INK4A}$ (12). To determine if hTERT is able to immortalize our HBEC, two different HBEC cultures were transfected with the pBABE-hygro-hTERT cDNA provided by Dr. Robert Weinberg. In the first experiment, both Type I and Type II HBEC of HME30 were transfected with the plasmids using lipofectin (4 μg DNA per 9 cm plate) and selected with hygromycin (10 μg/ml). While no colony was found for Type I cells (approximately 1 x 10^6 cells transfected), there are a total of 14 hygromycin-resistant colonies isolated from Type II cells (approximately 4 x 10^6 cells transfected) in addition to 5 plates with several colonies which were later subcultured together into 5-75 cm^2 flasks. The growth of these colonies or mixed colonies, however, stopped after one month in culture. In a second experiment, we used a HBEC

culture previously shown to be deficient in $p16^{INK4A}$ expression by Western blotting (HME15) (7). Approximately 6 x 10^6 Type II HBEC were transfected with the hTERT using similar procedure described above (Type I cells not available now for HME 15). A total of 23 hygromycin-resistant colonies were isolated. Although these colonies showed active proliferation initially, all colonies eventually ceased to proliferate. They are not considered as colonies with extended lifespan using SV40 transformed colonies as a criterion. The failure of these experiments to immortalize HBEC casts doubt about the consistency of this approach. The failure could also be due to the use of a different expression vector.

F. Mechanism of the Expression of ER in Type II HBEC by SV40 Large T-antigen (Task 6)

The normal Type II HBEC did not express the ER. The SV40 transformed Type II cells, similar to Type I cells, expressed the 48 kd variant ER when grown on plastic (3). The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen may induce the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast carcinoma cells with SV40. These cells expressing the large T-antigen resulted from SV40 transfection, however, remain ER-negative (3). Alternatively, in the Type II HBEC population, there might exist a small population of ER-positive transitional cells, newly differentiated from Type I cells, which are the target cells for SV40 transformation. Indeed, we have observed a

morphologically distinguishable atypical HBEC type which attached on plastic early similar to Type II cells after trypsinization and subculture. These cells are also very likely to be derived from Type I cells as they may be found in some Type I cell colony. Preliminary study shows that these cells have mixed phenotypes of Type I and Type II cells (i.e., not growth-inhibited by FBS, proficient in gap junctional intercellular communication and ER-positive). It is possible that a small minority of these cells were present in Type II cell population and they are the target cells transformable by SV40. We have done one experiment to transfect 2 plates of atypical HBEC colonies, after removing other types of colonies by scraping, with a plasmid carrying the wild type SV40 large T-antigen (PRNS-1) from Dr. Johng S. Rhim). The experiment, however, failed to produce the transformed colony.

In light of the results from the mutant p53 experiment which yields ER positive cells from Type II cells, the expression of ER in transformed Type II cells could be the result of dedifferentiation of ER-negative progenitor cells that are being transformed.

G. The Biological Functions of Estrogen Receptors Expressed in Normal HBEC (Task 7)

Only Type I HBEC expressed the ER. When grown on plastic, these cells expressed the 48 kd variant ER which appears to be non-functional in DNA-binding (3) and not responsive to estrogen for cell growth (2). What is not known is whether the wild type ER is expressed in

budding/ductal structures formed in Matrigel and responsive to estrogen for growth. This has not been done. The techniques for sectioning of organoids in Matrigel has been developed in the past year. This will be used in conjunction with immunostaining for this study.

H. The Biological Functions of Estrogen Receptors Expressed in SV40 Large T-antigen and X-ray Transformed HBEC (Task 8)

We have developed and characterized SV40 and x-ray transformed Type I HBEC lines (3, 8). These cells expressed a 48 kd ER *in vitro* on plastic and the wild type ER and a 51 kd ER in tumors formed in nude mice and in cells embedded in Matrigel (see report on Task 3 and reference 3). The 48 kd ER expressed in cells on plastic appears not to respond to estrogen to stimulate cell growth (Fig. 19 in 1998 Annual Report). Additional experiments show that these neoplastically transformed Type I HBEC (M13SV1R2N1) (8) also did not respond to estrogen for growth on plastic plates coated with different extracellular matrix components (collagen I, IV, fibronectin and laminin) (Biocoat Cellware, Collaborative Biomedical Products) (Fig. 8 in 1999 Annual Report). The Matrigel also seems not to modify the estrogen response for growth when these cells were inoculated on top, in between of two layers (overlay) or mixed (embedded) with Matrigel (Fig. 9 in 1999 Annual Report). This grant did not seek approval for animal experiments. Previous experiments carried out under a different grant (NIEHS

ES07256) show that M13SV1R2N1 formed tumors in both male and female mice (8) and in ovariectomized nude mice with or without estrogen pellet (Table 3 in 1999 Annual Report).

All these experiments indicate that the *in vitro* transformed cells are non-responsive to estrogen for growth *in vitro* and do not require estrogen for tumor growth despite the expression of the wild type ER. Therefore, we suspect the non-responsiveness of these cells to estrogen could be the result of their transformation by SV40 large T-antigen. To test this hypothesis, we have transfected the ER-positive, estrogen-dependent breast cancer cells MCF-7. The results, indeed, show that the response of SV40 large T-antigen expressing MCF-7 cells have partially reduced their response to estrogen for cell growth *in vitro* (Fig. 10 in 1999 Annual Report).

I. Correlation of High Susceptibility of a Normal HBEC Type with Stem Cell Characteristics to Neoplastic Transformation with its High Potential for Telomerase Activation (Task 9)

This Task has been completed. A detailed report was presented in the 1998 Annual Report. A paper was published in *Cancer Research* (W. Sun, K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Research* 59: 6118-6123, 1999).

Figure Legends

Figure 1 Spherical structures formed in Matrigel by Type II HBEC (HME 23) in 16 days.

Figure 2 The structure of spherical balls formed by Type II normal HBEC on Matrigel as revealed by sectioning. The structure is similar to squamous metaplasia developed by rat mammary organoid in Matrigel.

Figure 3 RT-PCR analysis of the expression of ER-α transcript. The primer pair used is E1U new (5′ CCT ACT ACC TGG AGA ACG AG 3′) and E5L (5′ CTG TCC AAG AGC AAG TTA GG3′). The 850 bp transcript is present in the positive control, the transformed Type I HBEC (M13SV1R2-2) and the MCF-7 cells but not in the ER-α-negative MDA-MB-231 cells. Two splicing variant transcripts are also found in M13SV1R2-2.

Figure 4 RT-PCR analysis of the usage of the 3 ER-α promoters in normal and transformed HBEC. The primer pairs are listed for promoter A, B and C.

Upstream Primers

ER1 5′ GCA CAG CAC TTC TTG AAA AAG G 3′

ER2 5′ TAC AGC TTT CTC TGG CTG TGC CA 3′

ER4 5′ CCT CGG GCT GTG CTC TTT TTC C 3′

Downstream Primer

ER5 5′ AGG GTC ATG GTC ATG GTC CG 3′

Table 1. Major phenotypic differences between Type I and Type II HBEC

	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobble-stone appearance
Colony morphology	Boundary smooth and restricted	Boundary not smooth
Attachment on plastic surface after trypsinization	Late	Early
Effect of FBS	Growth promotion	Growth inhibition
Gap junctional intercellular communication	Deficient	Efficient
Expression of:		
Connexin 26	–	+
Connexin 43	–	+
Epithelial membrane antigen	+	–
Cytokeratin 18	+	–
Cytokeratin 19	+	–
Cytokeratin 14	–	+
α6 Integrin	–	+
Estrogen receptor	+	–
Effect of cAMP (induced by cholera toxin, forskolin)	Induces Type I cells to change into Type II cells	
Organoid on Matrigel	Budding/ductal structure Acini	Spherical and elongated structures
Anchorage independent growth	+ (small colony)	–
Response to SV40 large T-antigen:		
Anchorage independent growth	– (large colony)	–
Immortalization	High frequency	Low frequency

Table 2. Extended Lifespan Clones Isolated from HME21 Transfected by m p53/c-myc

Exp. 1

	cpdl	AIG	ER	GJIC
M21-22	62	+	+	+
M21-23	44		+	
M21-24	58	+	+	+
M21-32	95	+	+	–
M21-41	40			
M21-73	38			
M21-82	39		–	+
M21-104	36		+	

Exp. 2

	cpdl	AIG	ER	GJIC
M21-B1	38			
M21-B2-1	37			
M21-B2-2	48	+	+	+

cpdl = cumulative population doubling level; AIG = anchorage independent growth; ER = estrogen receptor; GJIC = gap junctional intercellular communication

Figure 1.

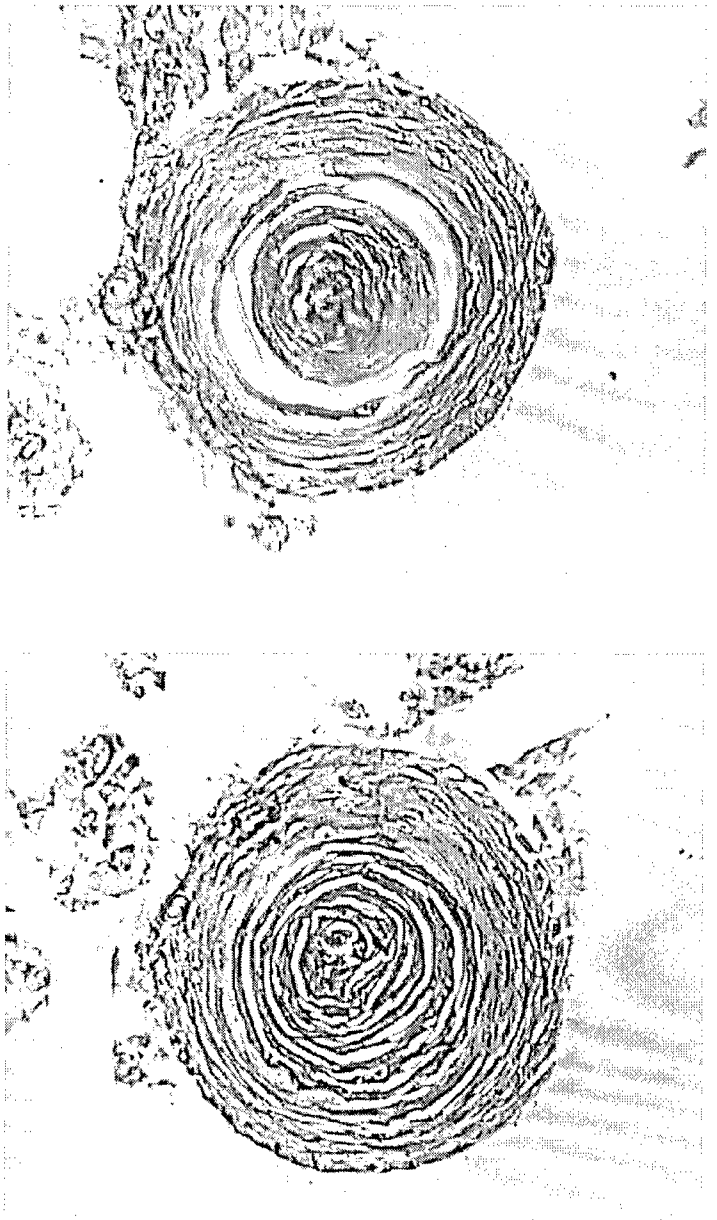
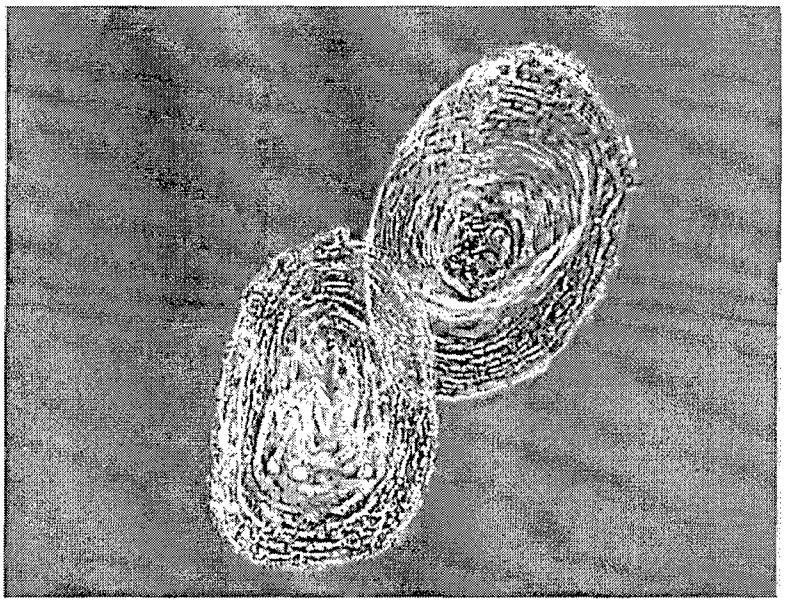


Figure 2.



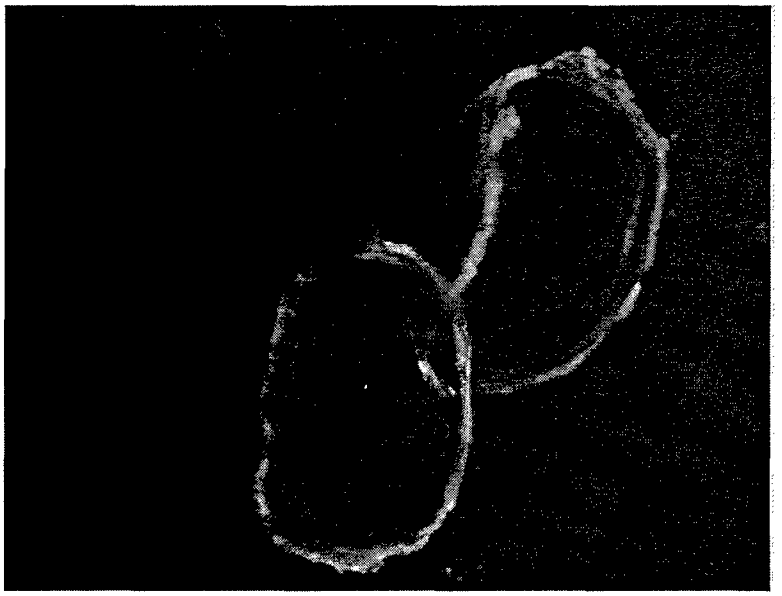
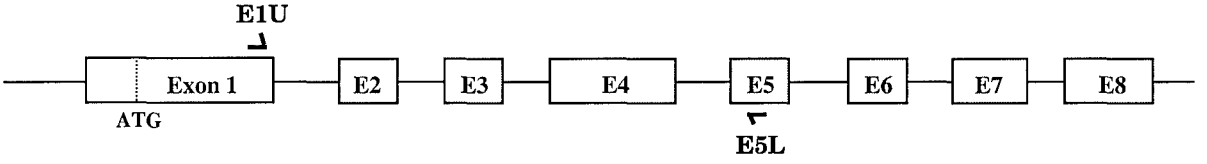


Figure 3.

RT-PCR analysis of ER-alpha transcripts

ER-alpha gene structure and RT-PCR primer locations



	W/T	~E2	~E3	~E4	~E2+E3	~E2+E4	~E3+E4	~E2+E3+E4
E1U/E5L	850bp	658bp	733bp	514bp	541bp	322bp	397bp	205bp

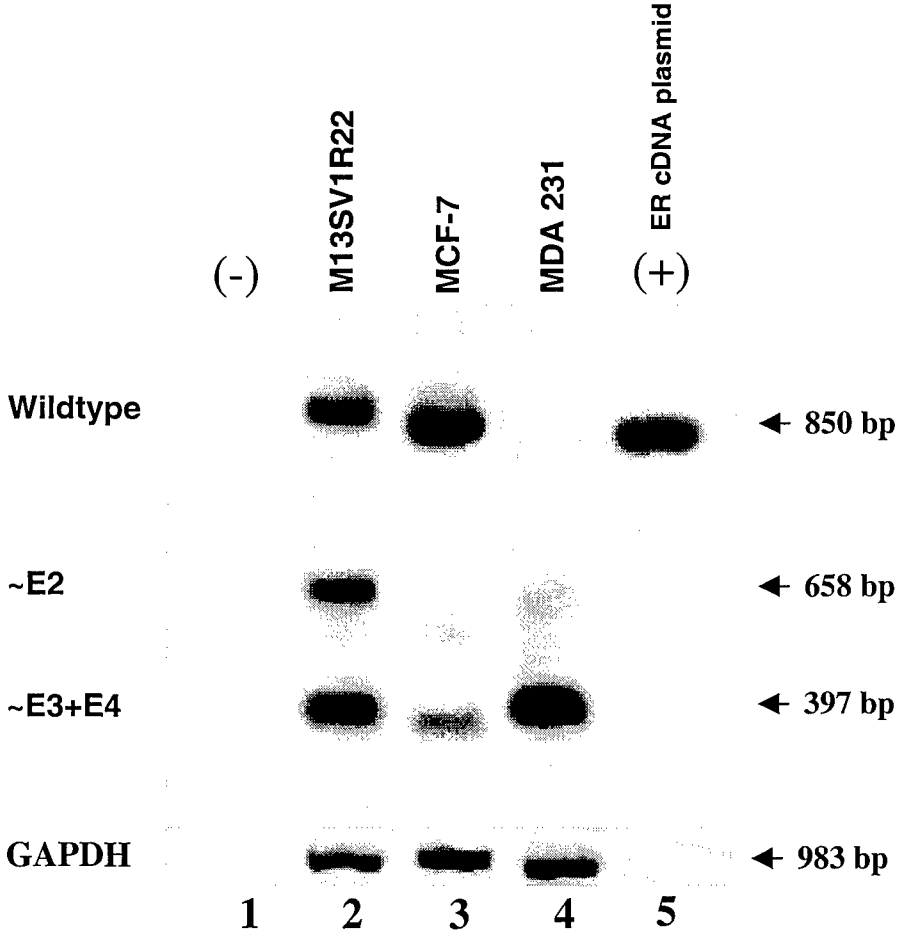
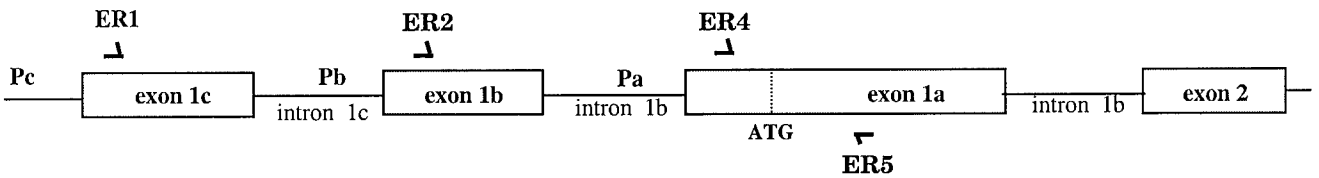


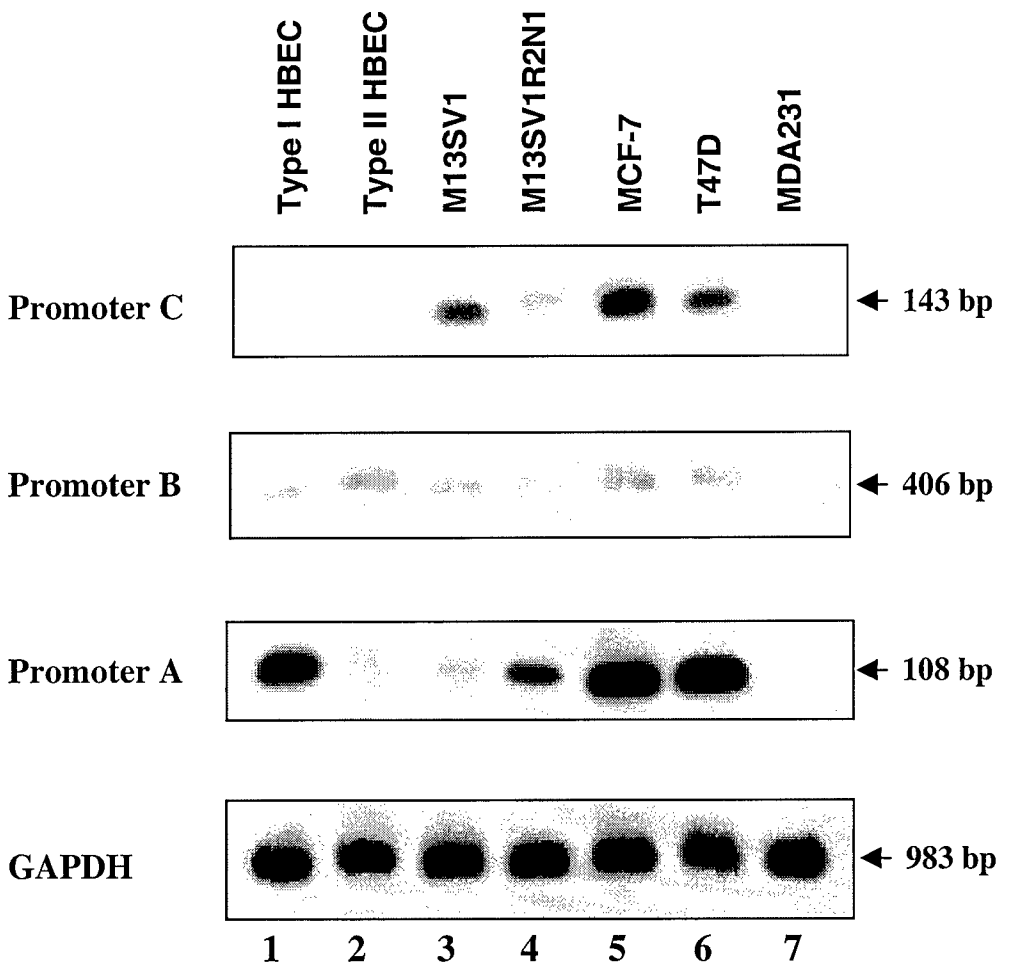
Figure 4.

Different Usage of the Three ER-alpha Promoters in Normal and Transformed HBECs

Partial Genomic Map of ER-alpha DNA and Primer Locations:



	Primers	Size of the RT-PCR product
Promoter C	ER1/ER5	143 bp
Promoter B	ER2/ER5	406 bp
Promoter A	ER4/ER5	108 bp



VII. KEY RESEARCH ACCOMPLISHMENTS

1. The discovery that normal Type I human breast epithelial cells (HBEC) and their neoplastically transformed derivatives express a variant ER (~48 kd) when grown *in vitro* on plastic and that tumors formed by these cells in nude mice expressed a wild type ER (~66 kd) and a different variant ER (~51 kd).
2. Demonstrated that Matrigel is able to mimic the *in vivo* condition to activate the expression of the wild type ER.
3. Obtained further evidence that Type I HBEC have stem cell characteristics, i.e., the ability to form budding/ductal structures and to maintain the undifferentiated state in Matrigel for a long time, in addition to previous observations that Type I cells are capable of differentiating into other types of cells.
4. The demonstration that Type I cells are more susceptible to telomerase activation and immortalization following transfection by SV40 large T-antigen (i.e., Type I cells are target cells for breast carcinogenesis).
5. Demonstrated that Type I HBEC can be neoplastically transformed by few steps of sequential treatments: transfection with SV40 large T-antigen (immortalization), x-ray irradiation (weakly tumorigenic), C-erbB2/neu (highly tumorigenic).
6. The development of an effective method to extend the lifespan (bypassing senescence) or to immortalize HBEC which express the ER

and are capable of anchorage independent growth by co-transfection with a dominant-negative p53 and human c-myc.

VIII. REPORTABLE OUTCOMES

A. Published Papers

1. Kang, K.S., I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang, 1997. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* 18: 251-257.
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IX. CONCLUSIONS

Our characterization of organoids formed by the two types of HBEC on Matrigel provides strong evidence that Type I HBEC are breast epithelial stem cells. The Type I cells not only are capable of differentiating into other cell type (1) and forming budding/ductal structures on Matrigel (4, 7), the organoids thus formed are also able to preserve HBEC for a long time. The

alternate cycling of the two types of HBEC as monolayer culture on plastic and as organoid in Matrigel could be a method to preserve and amplify human breast epithelial stem cells for other research on human breast epithelial stem cells. This organoid system should be useful as an *in vitro* model for analyzing factors that affect normal and abnormal growth and differentiation of human mammary gland and for studying the mechanism of carcinogenesis.

Our study of telomerase activity in normal and neoplastically transformed HBEC clarifies two issues regarding the role of telomerase in breast carcinogenesis. First, both normal Type I and Type II HBEC did express a low level of telomerase activity. However, this low level of telomerase activity may not be sufficient for neoplastic transformation. Second, in all the SV40 immortalized HBEC clones assayed, the telomerase activity was invariably greatly activated at mid- or late passage, in contrast to senescent clones which failed to activate the telomerase. Furthermore, the ability of Type I HBEC to become immortal at high frequency (11/11) appears to be correlated with their high potential for telomerase activation. Therefore, telomerase activation could be a major biomarker and mechanism for the transition from extended lifespan to immortalization, contrary to the conclusion of a previous report (6) that telomerase activity is a biomarker of cell proliferation but not malignant transformation. The high potential of Type I HBEC to activate telomerase activity also provides a mechanism why

Type I cells might be target cells for neoplastic transformation. If Type I stem cells are target cells for carcinogenesis, they should be also target cells for chemoprevention. Therefore, we hypothesize that agents capable of inducing differentiation and/or preferentially arrest the self-renewal of breast epithelial stem cells, thereby reducing the number of target cells for carcinogenesis, are potential chemopreventive agents for breast cancer. The hypothesis is supported by our Type I stem cell experiments mentioned above and animal experiments with genistein that induced mammary gland differentiation and reduced mammary tumorigenesis (13) as well as by epidemiological evidence that early full-term pregnancy reduces, while nulliparous or late parous increases, breast cancer (14-16), and our preliminary results indicating that potential chemopreventive agents, genistein and 1,25-dihydroxy-vitamin D_3, induced differentiation of breast epithelial stem cells (17).

The co-transfection with a temperature-sensitive mutant p53 and the human c-myc appears to be a very effective method to transform normal HBEC. The phenotypes of the transformed clones such as AIG and ER expression are similar to our Type I HBEC transformed by SV40. It should be noted that except our SV40 transformed Type I HBEC, AIG and ER expression have not been reported by other immortalized HBEC in literature. This newly-developed method and the transformed clones should be very useful for carcinogenesis study. The fact that HBEC can be easily transformed by dominant-negative mutant p53 in addition to the correlation

of the 3 hereditary breast cancer syndromes to the defective p53 (i.e., p53 mutation in Li-Fraumeni Syndrome, the Ataxia telangiectasia gene product ATM that phosphorylates and activates p53 and the BRCA1 that activates the expression of p53 (18-19) strongly suggests p53 as a major focus for breast carcinogenesis.

Our tumorigenic Type I cells transformed by sequential treatment with SV40, x-rays and neu oncogene did not require estrogen for cell growth or tumor development. This could be due to the expression of SV40 large T-antigen as shown by the reduced sensitivity to estrogen for growth in T-antigen expressing MCF-7 cells. To confirm this hypothesis, we are testing whether Type I HBEC transformed by other means such as mutant p53 could be estrogen-dependent similar to MCF-7 cells.

Our finding of the differential expression of ER *in vitro* and *in vivo* indicates the importance of cellular environment in regulating the expression of ER. We further demonstrated that the *in vivo* effect may come from extracellular matrix components as shown by the ability of Matrigel to mimic the *in vivo* condition in inducing the expression of wild type ER. This might provide a new strategy to control the ER expression and the growth of estrogen-dependent breast cancer by modulating the structure and function of extracellular matrix.

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XI. APPENDICES

Reprints of published papers and manuscripts in press:

1. Sun, W., K.S. Kang, I. Morita, J.E. Trosko and C.C. Chang. High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Res.* 59: 6118-6123, 1999.
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High Susceptibility of a Human Breast Epithelial Cell Type with Stem Cell Characteristics to Telomerase Activation and Immortalization[1]

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ABSTRACT

We have recently characterized two types of normal human breast epithelial cells (HBECs) from reduction mammoplasty. Type I cells express estrogen receptor, luminal epithelial cell markers, and stem cell characteristics (*i.e.*, the ability to differentiate into other cell types and to form budding/ductal structures on Matrigel), whereas Type II cells show basal epithelial cell phenotypes. In this study, we have examined whether Type I HBECs are more susceptible to telomerase activation and immortalization after transfection with SV40 large T-antigen. The results show that both types of cells acquire extended life span [(EL); *i.e.*, bypassing senescence] at a comparable frequency. However, they differ significantly in the ability to become immortal in continuous culture, *i.e.*, 11 of 11 Type I EL clones became immortal compared with 1 of 10 Type II EL clones. Both parental Type I and Type II cells as well as their transformed EL clones at early passages [~30 cumulative population doubling level (cpdl)] showed a low level of telomerase activity as measured by the telomeric repeat amplification protocol assay. For all 11 of the Type I EL clones and the single Type II EL clone that became immortal, telomerase activities were invariably activated at middle passages (~60 cpdl) or late passages (~100 cpdl). For the four Type II EL clones randomly selected from the nine Type II clones that did not become immortal, the telomerase activities were found to be further diminished at mid-passage, before the end of the life span. Thus, normal HBECs do have a low level of telomerase activity, and Type I HBECs with stem cell characteristics are more susceptible to telomerase activation and immortalization, a basis on which they may be major target cells for breast carcinogenesis.

INTRODUCTION

Cancer cells are generally recognized as being in a relatively undifferentiated state and could arise from stem cells with blocked or partially blocked differentiation (1, 2). Epidemiological studies indicate that the lifetime risk of developing breast cancer in child-bearing women seems to be linearly related to the age at which a women has her first full-term pregnancy (3) and that breast cancer risk is higher in those who are nulliparous or late parous (4, 5). This has been hypothesized to be due to stem cell multiplication during each ovarian cycle before but not after the first pregnancy (6) or to the induction of mammary gland differentiation by pregnancy with the elimination of terminal end buds, resulting in refractoriness of the gland to carcinogenesis (7). Although the role of stem cells in cancer is implicated, whether stem cells are more susceptible to neoplastic transformation has not been examined experimentally.

Normal somatic cells have a finite life span. Telomere shortening as a consequence of the end-replication problem has been proposed as a mitotic clock for cellular senescence (8). Telomerase, a ribonucleoprotein complex with reverse transcriptase activity that uses a RNA template to add TTAGGG hexonucleotide repeat onto the end of chromosomes, is capable of maintaining the telomere length and replicative activity of cells (9). Whereas normal human cells or adult tissues, in general, lack telomerase activity, the great majority of human tumors from various tissues and immortal cell lines show telomerase activity (10, 11). A current predominant hypothesis proposes that the reexpression of telomerase occurs in most tumors and is probably a critical event responsible for continuous tumor cell growth (12), recognizing the existence of alternative mechanism for immortalization (13–15). Additional studies have revealed that telomerase activity is expressed in human germ-lines (testes and ovaries) (12), human embryonic stem cells (16), human lymphocytes and hematopoietic progenitor cells (17–20), candidate stem cells from the fetal liver (21), human epidermal cells expressing a basal cell marker (22), and from the basal layer (23) and in human endothelial (24) and uroepithelial cells (25). The latter two cell types showed proliferation-dependent expression of telomerase (24, 25). The role of telomerase in malignant transformation has been questioned by a recent study that observed the presence of telomerase activity in both normal and tumorigenic human cells including breast epithelial cells (25). However, this study did not quantitatively measure telomerase activity during the course of neoplastic transformation.

We have developed a cell culture method to grow two types of normal HBECs[4] from reduction mammoplasty (26). One type of cell (Type II HBECs), similar to those commercially available or used by most other laboratories, shows basal epithelial cell markers. The other cell type (Type I HBECs) expresses luminal epithelial phenotypes and estrogen receptors (26, 27). Importantly, Type I HBECs also show stem cell characteristics (*i.e.*, the ability to differentiate into other cell types by cyclic AMP-inducing agents and to form budding/ductal structures in Matrigel). Because telomerase activity in human stem cells has not been well studied, our first objective is to determine whether telomerase is expressed in our two types of cells. Furthermore, it offers an opportunity to examine whether the HBECs with stem cell characteristics (Type I HBECs) are more susceptible to telomerase activation and immortalization.

MATERIALS AND METHODS

Cell Culture and Mammary Organoids Formation. The cell culture and the method to develop the two types of normal HBEC culture from reduction mammoplasty were as described previously (26). The two types of HBECs are morphologically distinguishable and substantially different in many phenotypes (26). In contrast to Type II cells, the major features of type I HBECs are the deficiency in gap junctional intercellular communication, the growth promotion by fetal bovine serum, and the expression of estrogen receptors and luminal epithelial cell markers (*i.e.*, epithelial membrane antigen and cytokeratin 18; Refs. 26–28).

Growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) was used to study mammary organoid structure formation with Type I and Type II HBECs. Approximately, 1–1.5 × 10^6 cells were plated on 35-mm culture dishes or two-chamber Lab-Tek culture slides with a layer of Matrigel. Matrigel remains solid in the 37°C humidifed incubator where cells were

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[4] The abbreviations used are: HBEC, human breast epithelial cell; EL, extended lifespan; cpdl, cumulative population doubling level; TRAP, telomeric repeat amplification protocol; AIG, anchorage-independent growth; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

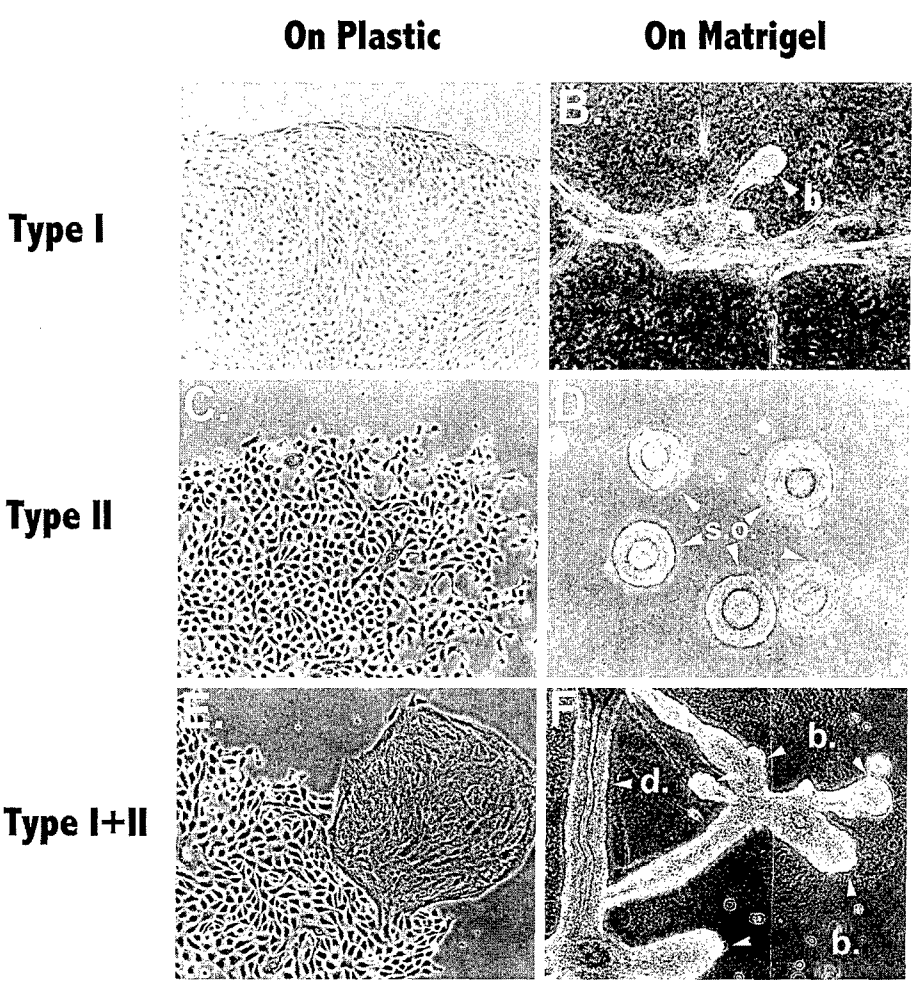

Fig. 1. HBEC colonies on plastic and organoids on Matrigel formed by two types of normal HBECs. Type I and Type II colonies developed on plastic (*A* and *C*, respectively) are morphologically distinguishable. On Matrigel, Type II cells typically formed spherical organoid (*s.o.*) (*D*), whereas Type I cells formed a limited number of bud-like (*B*) structures and acini (not shown). The combination of Type I and Type II cells (*E*; two types of cells on plastic) in 1:2 or 1:3 ratios can generate many budding (*B*)/ductal (*D*) structures in Matrigel (*F*) in 2–3 weeks.

allowed to aggregate for 1 day. After cells attached to Matrigel, the medium was changed or a second layer of Matrigel was placed on top of the first layer. Culture medium was changed once every 2 days.

Development of SV40 Large T-Antigen-transformed Type I and Type II HBECs. The normal HBECs were transfected with a plasmid carrying an origin-defective SV40 genome expressing a wild-type large T-antigen [PRNS-1; a gift from Johng S. Rhim, Uniformed Services University of the Health Sciences, Bethesda, (M15SV1–11 and M15SV21–30 were derived from Type I and Type II HME 15, respectively)] by Lipofectin (Life Technologies, Inc., Gaithersburg, MD). The actively proliferating colonies were selected by their resistance to G418 (0.4 mg/ml for M15SV1–11 from Type I HBECs and 0.15 mg/ml for M15SV 21–30 from Type II HBECs). The proliferation potential of transformed clones was determined by their total cpdls using the formula cpdl = ln(*Nf*/*Ni*)/ln 2, where *Ni* and *Nf* are initial and final cell numbers, respectively, and ln is the natural log. The initial cell number was 2×10^5 for each propagation.

During the course of determining the potential cpdl for each SV40-transformed cell line, the populations of cells at different cpdls were preserved in liquid nitrogen. For the telomerase assay, the cells at early (22–30 cpdl), middle (50–60 cpdl), and late (100–110 cpdl) passages were grown and harvested to prepare cell lysates.

PCR-based Telomerase Assay. Cells grown to about 50–70% confluence were harvested by trypsinization. After cell counts, the cells were centrifuged to remove trypsin solution. The cell pellet for each culture was washed with 10 ml of PBS and then centrifuged to remove PBS. Cells were then suspended at 1×10^6 cells/ml in PBS and aliquoted to Eppendorf tubes. After cells were centrifuged and PBS was carefully removed, the cell pellets were stored at −85°C. For the telomerase assay, the cell pellet was thawed and resuspended in 200 μl of 1× CHAPS lysis buffer/10^6 cells and left on ice for 30 min. The samples were spun in a microcentrifuge at 12,000 × *g* for 20 min at 4°C. The cell lysate for each sample was aliquoted to several new tubes and stored at −85°C. The original lysate represents a concentration of 5,000 cells/μl. Further dilution of the cell lysate was adjusted based on the level of telomerase activity for the individual cell line. Telomerase activity was examined by the TRAP assay (10) using the TRAPez Telomerase Detection Kit (Oncor, Gaithersburg, MD). This protocol includes primers of a 36-bp internal control (I.C.) for quantitating the amplification efficiency, thus providing a positive control for accurate quantitation of telomerase activity within a linear range close to 2.5 logs. Each analysis included a negative control (CHAPS lysis buffer instead of cell lysate), a heat-inactivated control (the sample was incubated at 85°C for 10 min before the assay), and a positive cell line control (breast carcinoma cell line MCF-7). For RNase treatment, 10 μl of extract were incubated with 1 μg of RNase for 20 min at 37°C. The products of the TRAP assay were resolved by electrophoresis in a nondenaturing 12% PAGE in a buffer containing 54 mM Tris-HCl (pH 8.0), 54 mM boric acid, and 1.2 mM EDTA. The gel was stained with Syber Green (Molecular Probes, Inc., Eugene, OR) and visualized by a 302-nm or 254-nm UV transilluminator. Images were captured and analyzed by AlphaImager (Alpha Innotech Corp., San Leandro, CA) or NucleoVision760 (NucleoTech Corp., San Mateo, CA). Quantitation of the products generated from TRAP assay was calculated using the following formula: *TPG* (total product generated units) = $((x - x_o)/c)/((r - r_o)/c_R) \times 100$, where x and x_o represent signals corresponding to the TRAP product ladder bands of non-heat-treated and heat-treated sample lanes, respectively, r and r_o represent signals from 1× CHAPS lysis buffer control (*i.e.*, primer-dimer/PCR contamination control) and TSR8 (DNA quantitation control), respectively. c and c_R are the signal from the internal standard (TSK1) in non-heat-treated samples and the signal from TSR8 quantitation control, respectively.

Table *Telomerase activity in SV40 large T-antigen-transformed Type I and Type II HBECs at different cpdls*

Cell line	Parental cell type	Telomerase activity at different passage levels[a]			Immortalized (cpdl)[b]
		Low	Middle	High	
M15SV1	Type I	ND	++++	++++	Yes (>110)
M15SV2	Type I	+	+	++++	Yes (>106)
M15SV3	Type I	+	+	++++	Yes (>107)
M15SV4	Type I	+	++++	++++	Yes (>103)
M15SV5	Type I	+	+++	+++	Yes (>105)
M15SV6	Type I	+	++++	++++	Yes (>107)
M15SV7	Type I	+	++++	++++	Yes (>102)
M15SV8	Type I	+	++++	++++	Yes (>109)
M15SV9	Type I	+	++++	++++	Yes (>113)
M15SV10	Type I	+	++++	++++	Yes (>103)
M15SV11	Type I	+	++++	++++	Yes (>104)
M15SV21	Type II	+	±	(Senescent)	No (32)
M15SV24	Type II	+	±	(Senescent)	No (76)
M15SV26	Type II	+	±	(Senescent)	No (39)
M15SV27	Type II	+	ND	(Senescent)	No (31)
M15SV29	Type II	+	±	(Senescent)	No (62)
M15SV30	Type II	ND	++++	ND	Yes (>107)

[a] ±, +, and ++++ denote very weak, low, and high telomerase activity, respectively; ND, not done.
[b] The cpdl for other Type II clones are as follows: M15SV22, 32; M15SV23, 37; M15SV25, 39; and M15SV28, 43.

RESULTS

Stem Cell Characteristics of Type I HBECs as Indicated by Organoid Formation and Growth. Previously, we have shown that Type I HBECs have the ability to differentiate into Type II HBECs by cyclic AMP-inducing agents (cholera toxin and forskolin; Refs. 26 and 29). Additional evidence that Type I cells have stem cell characteristics came from the study of organoid formation and growth on Matrigel. When Type I and Type II cells were plated separately on top of Matrigel or in between two layers of Matrigel, Type I cells characteristically formed acinar structures that are formed by luminal epithelial cells as shown previously (30) and onganoid showing some limited budding structure formation (Fig. 1*B*), whereas Type II cells with basal epithelial phenotypes formed a spherical organoid (Fig. 1*D*). When the two types of cells were plated together on Matrigel, they formed a ductal and terminal end bud-like structure (Fig. 1*F*). Because mammary stem cells are known to be present in the end bud for ductal morphogenesis and elongation (31, 32), the ability of Type I HBECs to form these structures strongly indicates that the Type I HBEC population contains mammary epithelial stem cells that are capable of giving rise to luminal and basal epithelial cells.

High Susceptibility of Type I HBECs to Immortalization. Both Type I and Type II HBECs were transfected with an origin-defective SV40 genome expressing the wild-type large T-antigen (PRNS-1) and selected by G418. Eleven independent clones were isolated from ~3 × 10^6 Type I HBECs, whereas 10 clones were derived from ~4 × 10^6 Type II HBECs. All these Type I and Type II clones were able to bypass senescence and acquire an EL (*i.e.*, more than 30 cpdl; normal HBECs never grew more than 20 cpdl). Therefore, the ability of SV40 large T-antigen-transformed Type I and Type II HBEC clones to acquire EL appears to be comparable. However, the frequency at which EL clones became immortal differs significantly between Type I and Type II HBEC-derived clones. All of the 11

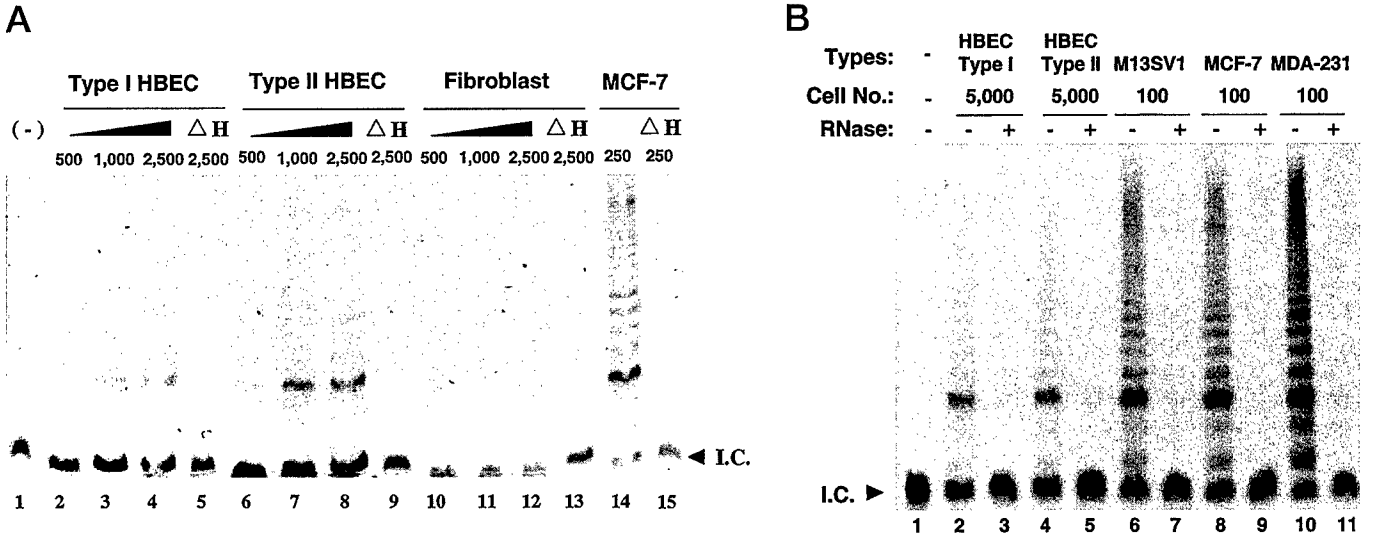

Fig. 2. Telomerase activity in Type I and Type II HBECs at passage two. *A*, telomerase activity was detected in cell lysates derived from different cell numbers as shown by a PCR-based TRAP assay, as described in "Materials and Methods." *Lane 1* and *Lanes 5, 9, 13*, and *15* represent negative controls without cell lysate (−) and heat-inactivated controls (Δ *H*), respectively. The Type I HBECs, Type II HBECs, and fibroblasts used in this assay were all derived from the mammary tissue of one patient. As a positive control, the breast carcinoma cell line MCF-7 showed a high level of telomerase activity (*Lane 14*, 250 cells). Low levels of telomerase activity were detected in both normal Type I (*Lanes 2–4*) and Type II (*Lanes 6–8*) HBECs; the activity in fibroblasts was undetectable (*Lanes 10–12*). *B*, RNA dependency of the weak telomerase activity detected from primary Type I and Type II HBECs (lysate from 5000 cells), and the high activity from SV40-immortalized Type I HBECs (M13SV1) and two breast cancer cell lines (MCF-7 and MDA-MB-231). The extract equivalent to 100 cells of lysate was used in each assay. *I.C.*, the internal control for quantitating the amplification efficiency in the TRAP assay.

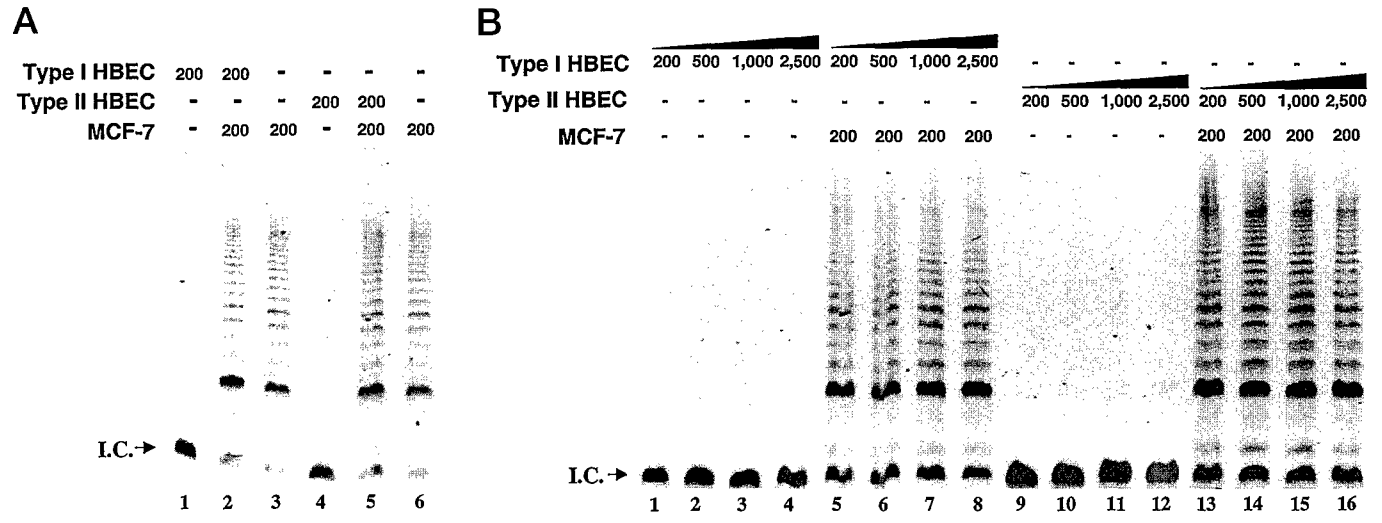

Fig. 3. Absence of telomerase inhibitor in Type I and Type II HBECs. Telomerase activity from a mixture of telomerase-positive MCF-7 cell lysate and HBEC lysate was examined to clarify that low levels of telomerase activity in both types of HBECs were not due to the presence of telomerase inhibitors in these cells. The numbers represent an equivalent amount of cells in the lysate. *A* and *B* are two independent experiments. No significant difference in telomerase activity was found between MCF-7 alone and the mixed lysates. *I.C.*, the internal control in the TRAP assay.

(100%) EL clones derived from Type I cells have become immortal (*i.e.*, actively proliferating after more than 100 cpdl). In comparison, only 1 of 10 (10%) EL clones derived from Type II cells has become immortal (Table 1). Excluding the immortal clone, the average life span of EL clones from Type II HBECs was 43 ± 5 cpdl. It is clear that Type I HBECs with stem cell characteristics were more susceptible to immortalization after SV40 large T-antigen transfection.

Telomerase Activity in Primary HBECs. To study the potential mechanisms underlying the high susceptibility of Type I HBECs to immortalization, we first studied the telomerase activities in primary Type I and Type II HBECs without SV40 transfection. As shown in Fig. 2*A*, telomerase activity was present in both Type I (*Lanes 2–4*) and Type II (*Lanes 6–8*) HBECs. However, the level of activity was weak compared with that of the breast carcinoma cell line MCF-7 (*Lane 14*). Using one-tenth of the cell number in the assay (250 *versus* 2500 cells), the telomerase activity in MCF-7 was significantly higher than that in Type I and Type II HBECs. In contrast, telomerase activity was undetectable in human breast stromal fibroblasts at early passages (*Lanes 10–12*). The telomerase activities in normal and in immortal or tumorigenic HBECs are dependent on enzyme activity from the catalytic subunit and RNA template, as shown by the elimination of the activity by heat inactivation or RNase treatment (Fig. 2, *A* and *B*). To exclude the possibility that the low level of telomerase activity from primary HBECs may be due to the existence of potential telomerase inhibitors, assays with mixed cell lysates from both HBECs and MCF-7 were carried out (Fig. 3, *A* and *B*). Because HBECs did not affect the telomerase activity of MCF-7 cells in the lysate mixtures, no telomerase inhibitor was detected in either Type I or Type II HBECs.

The Weak Telomerase Activity in Normal HBECs Is Insufficient for Immortalization. Because a low level of telomerase activity was found in primary HBECs, it is important to study whether further telomerase activation is required for these cells to become immortal. We have investigated the telomerase activity during the course of cell passage toward crisis or immortalization (Fig. 4, *A* and *B*). The same level of telomerase activity was found in both early-passage (before 50 cpdl) SV40-transformed Type I and Type II clones as in normal HBECs. There was a dramatic increase of the activity around 50–60 cpdl for clones that became immortal, and the activity remained high thereafter. In contrast, Type II EL clones without telomerase activation always stopped proliferating at crisis, as shown later (Fig. 6). The fact that the activated levels of telomerase activity

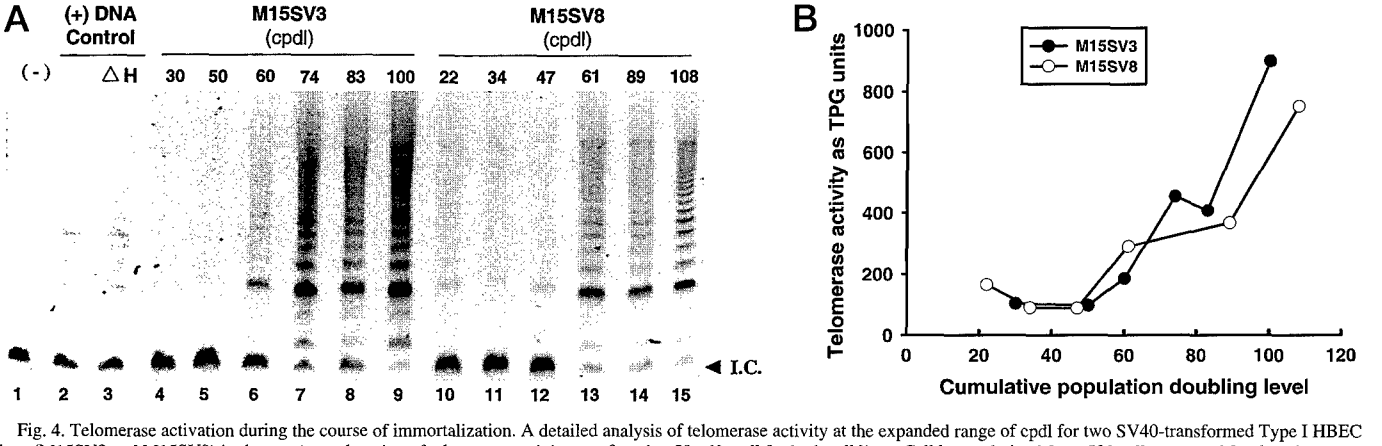

Fig. 4. Telomerase activation during the course of immortalization. A detailed analysis of telomerase activity at the expanded range of cpdl for two SV40-transformed Type I HBEC lines (M15SV3 and M15SV8) is shown. *A*, an elevation of telomerase activity was found at 50–60 cpdl for both cell lines. Cell lysate derived from 500 cells was used for the telomerase assay. *I.C.*, the internal control in the TRAP assay. *B*, quantitative measurement of telomerase activity in *A*. Telomerase activity was measured as total product generated (*TPG*), as described in "Materials and Methods."

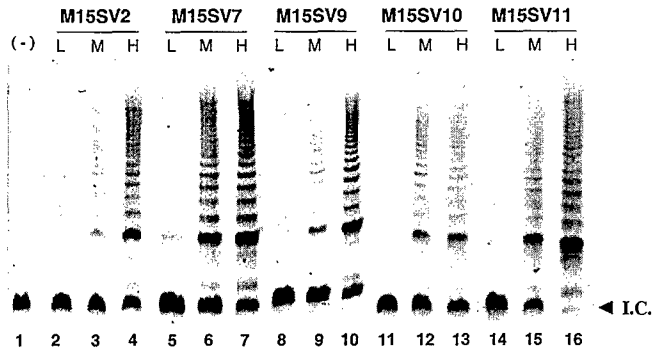

Fig. 5. Telomerase activity in SV40-transformed Type I HBEC-derived cell lines. Five SV40-transformed cell lines (M15SV2, M15SV7, M15SV9, M15SV10, and M15SV11), which became immortal, were examined for telomerase activity at low passage (*L*; ~22–30 cpdl), middle passage (*M*; ~50–60 cpdl), and high passage (*H*; >100 cpdl). In all of these cell lines, telomerase activities were elevated at middle or late passage. Cell lysate derived from 500 cells was used for the telomerase assay for each sample. *I.C.*, the internal control in the TRAP assay.

were found in all immortal cell lines and the fact that the majority of Type II EL clones did not become immortal indicate that the low level of telomerase activity detected in normal HBECs is insufficient for immortalization.

High Susceptibility of Type I HBECs to Telomerase Activation. Cell populations harvested at early passage (EL, ~30 cpdl), middle passage (around the potential crisis period, ~50–60 cpdl), and late passage (immortalization; >100 cpdl), respectively, were assayed. All 11 Type I HBEC-derived clones showed a significant increase in telomerase activity at middle or late passage (Table 1; only the results from seven clones are shown in Figs. 4 and 5). However, among Type II HBEC-derived clones, the only one that became immortal has shown activated telomerase activity (Fig. 6). Four of the nine Type II clones that did not become immortal were randomly selected for the telomerase activity assay. None of these clones showed telomerase activation at mid-passage before the advent of crisis. As summarized in Table 1, all Type I and Type II clones with activated telomerase activity eventually became immortal. These results suggest that Type I HBEC clones have a high potential for telomerase activation, which results in a high rate of immortalization.

DISCUSSION

The major findings of this study are as follows: (*a*) Type I HBECs were more susceptible to immortalization (11 of 11 clones) than Type II HBECs (1 of 10 clones) after SV40 large T-antigen transfection; (*b*) both normal Type I and Type II HBECs had a low level of telomerase activity that was insufficient to maintain continuous cell proliferation unless it was activated; and (*c*) the high potential of telomerase activation for Type I HBECs resulted in a more efficient immortalization compared to Type II HBECs. Because Type I HBECs have stem cell characteristics, these results suggest that the aforementioned mechanism is the reason why stem cells are more likely to be target cells for neoplastic transformation.

We have previously shown that Type I and Type II HBECs differ substantially in their response to an oncogenic (SV40) stimulus; *i.e.*, Type I cells were AIG^+, whereas SV40-transformed Type II cells totally lack the ability to grow in soft agar (AIG^-; Ref. 26). We were able to confirm that the SV40-transformed Type I HBEC clones obtained in this study are capable of AIG (eight of eight clones[5]). Therefore, this study provides additional and stronger evidence that Type I HBECs are more susceptible to tumorigenic initiation by acquiring two major and common tumor cell phenotypes, *i.e.*, AIG^+ and immortality.

Whereas it is reasonable to expect Type I cells with stem cell characteristics to have telomerase activity, the presence of telomerase activity in Type II HBECs seems difficult to reconcile with previous reports that normal breast tissues (10) and normal HBECs (33) do not have telomerase activity. It is possible that our Type II cells are newly derived from Type I cells. Therefore, they could be considered as progenitor cells for basal epithelial cells. Indeed, type II cells can be derived from Type I cells after treatment with cyclic AMP-inducing agent (26). The newly derived cells are similar to early-passage Type II cells from breast tissue in cell phenotype and proliferation potential. The low telomerase activity in normal HBECs and early-passage SV40-transformed HBECs is not due to the presence of telomerase inhibitors, as demonstrated in Fig. 3, or to the lower proportion of cycling cells because all cell cultures were harvested at near log phase of cell growth. However, this low level of telomerase activity in both Type I and Type II cells seems insufficient for unlimited growth for SV40-transfected cells, as shown by the requirement of telomerase activation in immortalized clones and the incapability of most Type II EL clones to become immortal.

The ability of Type I HBECs to form budding/ductal structures strongly suggests the stem cell characteristics of a proportion of these cells. The similarity of phenotypes between breast carcinoma cells and Type I cells (*i.e.*, deficiency in gap junctional intercellular communication, expression of estrogen receptors, and luminal epithelial cell markers) further indicates that breast cancers could be derived from Type I cells as a result of blocked differentiation, consistent with the oncogeny as blocked or partially blocked ontogeny theory of carcinogenesis (2).

The differential susceptibility of different HBEC types to immortalization by human papilloma virus (34) and by the catalytic component of telomerase, hTERT (33), has been reported previously. None of these cells are similar to the Type I HBECs.

The high susceptibility of SV40-transfected Type I cells to immortalization may provide a basis for the idea that stem cells are major targets for neoplastic transformation. In turn, the high potential of susceptibility to telomerase activation seems to be a mechanism that Type I cells become immortal at higher rate than Type II HBECs. However, the mechanism that telomerase is more easily activated in

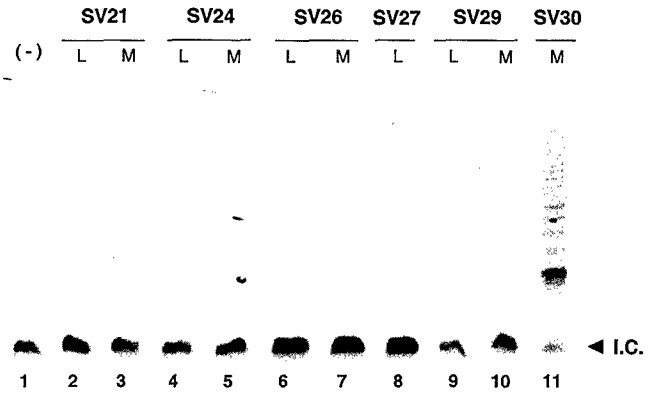

Fig. 6. Telomerase activity in SV40-transformed Type II HBECs. The majority of SV40-transfected Type II clones (9 of 10) did not become immortal beyond EL (Table 1). Five (M15SV21, M15SV24, M15SV26, M15SV27, and M15SV29) of these nine clones were analyzed for telomerase activity at low passage (*L*; ~25 cpdl) and mid-passage (*M*; ~40–50 cpdl). Telomerase activities in these clones diminished from low to middle passage when they were approaching crisis. Only the immortalized Type II HBECs (M15SV30) showed telomerase activation at middle passage (*Lane 11*). Cell lysate derived from 500 cells was used for each assay. *I.C.*, the internal control in the TRAP assay.

[5] Unpublished observations.

Type I cells remains unknown. Our results show that it is not due to a higher level of telomerase activity in Type I cells than Type II cells. In human tumor cells, the activity of telomerase has been shown to be cell cycle dependent, with the highest level of activity detected in S phase, and the lowest level found in cells arrested at G_2-M phase (35). Overexpression of cyclin D1 and/or cyclin E was a typical feature of breast cancers with high telomerase activity (36). It is likely that telomerase expression may be partially under the same control that regulates G_1 to S-phase transition. In this respect, our previous observation that the expression of cyclin D1 was higher in Type I HBECs than in Type II cells (27) may be relevant.

A previous report concludes that telomerase activity may not be a biomarker for malignant transformation because it is present in both normal and tumor cells (25). This conclusion may be misleading, because there is no quantitative comparison, and the comparison was made between tumor cells and unrelated normal cells. Using a well-characterized quantitative assay, we found that although both normal and immortal or tumorigenic cells have telomerase activity, the activities in immortal or tumorigenic cells are dramatically higher than that seen in normal cells. Indeed, the transition from low to high telomerase activity may be an indicator of when immortal cells are present in the population during the course of immortalization.

This study also has implications concerning the mechanism of carcinogenic initiation of breast epithelial cells. Because the function of SV40 large T-antigen is to inactivate p53 and pRb and to induce the CCAAT box binding factor that transactivates cell cycle-regulating genes such as cdc2 (37), alteration in cell cycle regulation seems to be the major event to acquire an EL for normal HBECs. The subsequent conversion of a cell with EL to an immortal cell clearly involves the activation of telomerase, as shown in this study. This is consistent with a recent report that both Rb/P16^{INK4a} inactivation and telomerase activity are required to immortalize HBECs (33).

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Roles of Ionising Radiation in Neoplastic Transformation of Human Breast Epithelial Cells

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Introduction

Although advances in breast cancer research have been made in recent years, the aetiology of breast cancer is not fully understood. However, a major role of lifetime exposure to oestrogen in breast cancer seems to be well established. This oestrogen–breast cancer theory is supported by the age–incidence curve which shows a lower rate of linear increase in breast cancer incidence after menopause (1) as well as by chemoprevention studies with the anti-oestrogen tamoxifen and the oestrogen substitute raloxifene, which reduced the risk of breast cancer. Many environmental agents might cause breast cancer by functioning as xenoestrogens or as oestrogen potentiating factors (2). While the evidence for these compounds is not yet substantial, ionising radiation is considered as the most well-established breast cancer carcinogen based on the excess risk of breast cancer associated with Japanese women exposed to atomic bomb radiation in Hiroshima and Nagasaki (3), as well as from radiation therapy of Hodgkin's disease (4) and other nonmalignant conditions (5).

Although the evidence that ionising radiation is a carcinogen for breast cancer is convincing, questions concerning its mechanisms of action remain to be elucidated. These include: a) At which stage of carcinogenesis does the agent exert its effect? b) Is breast cancer caused by genetic and/or epigenetic effects of ionising radiation? c) Which genes are affected by the agent and responsible for neoplastic transformation? d) Are there target cells in mammary glands that specifically respond to ionising radiation to induce breast cancer? The latter is related to the role of stem cells and differentiation in carcinogenesis. This paper addresses these questions and suggests an in vitro cell culture system for these studies.

Ionising Radiation Affects Different Stages of Carcinogenesis

From in vitro studies, ionising radiation has been shown to immortalise or neoplastically transform normal human fibroblasts (6, 7) and keratinocytes (8) and to induce neoplastic conversion of immortalised human epidermal keratinocytes (9). For human breast epithelial cells, repeated γ irradiation (2 Gy $\times$ 15) has been reported to produce an immortal cell line (10). These cells, although unable to grow in soft agar, were able to form tumours in nude mice.

An immortal nontumourigenic cell line, derived from a normal human breast epithelial cell (HBEC) type with luminal and stem cell characteristics after transfection with SV40 large T antigen, can be converted by X rays (2 Gy $\times$ 2) into weakly tumourigenic cell lines which were selected as large fast-growing colonies in soft agar (11). These weakly tumourigenic cells, but not the parental immortal cells, responded to the ectopic expression of a mutated *neu* oncogene to enhance their tumourigenicity in nude mice. Although ERBB2 (also known as $p185^{C\text{-}erbB2}$) neu proteins are highly expressed in both immortal and X-ray transformed tumourigenic cell lines after the infection with the *neu* oncogene, only in the latter were the

ERBB2/neu proteins tyrosine phosphorylated (11). The mechanism for the differential response is not known. One possible mechanism is that a protein tyrosine phosphatase is present in the immortal cell line but not in the weakly tumourigenic cell lines transformed by X rays. The possible deletion of a protein tyrosine phosphatase gene such as the *PTEN* tumour suppressor (12) is to be investigated. In a different series of experiments, we found that normal human breast epithelial cells can be converted to cell lines with extended life span (i.e. bypassing senescence). It is clear that ionising radiation is capable of extending the life span, immortalising and developing tumourigenicity in HBECs.

Genetic and Epigenetic Effects of Ionising Radiation on Breast Carcinogenesis

Besides being a well-known physical mutagen, ionising radiation has been shown to induce the expression of a variety of genes including DNA-binding transcription factors, growth factor and growth-related genes, proto-oncogenes, signal transduction and DNA repair genes (13–14). The importance of these effects on radiation-induced breast cancer remains to be determined.

Ataxia telangiectasia (AT) heterozygotes, who make up about 1% of the general population, have a 5.1-fold excess risk of breast cancer in women (15). The product of the *ATM* gene, which is mutated in patients with AT, has been shown to phosphorylate IKβ-α (16) and TP53 (17). This suggests a role of the *ATM* gene in NFKB activation and TP53 activation and cell growth control (16). Furthermore, AT cells were found to be more susceptible to the transcriptional activation of MYC and XRCC1 than other human cells after X irradiation (18).

Genes Affected by Ionising Radiation and Related to Neoplastic Transformation

Many genes with diverse functions have been shown to be inducible by ionising radiation (13, 14). These genes may be involved in cellular recovery and adaptation or could trigger signal transduction and transcriptional activation related to tumour initiation, promotion or metastasis.

There are only a few genes that are known to be mutated by ionising radiation and shown to be important for neoplastic progression. The *TP53* tumour suppressor gene appears to be the best known. Skin fibroblast cultures, derived from Li-Fraumeni syndrome, did not become immortal in culture but have been immortalised by X irradiation (one of six cultures) (19). These results suggest that loss of the wild-type *TP53* gene was necessary but not sufficient for immortalisation. It is interesting that the only human breast epithelial cell line immortalised by γ irradiation also lacked the TP53 tumour suppressor protein (10). However, the *TP53* mutation may not be the earliest tumour initiation event, since *TP53* mutations have been found in mammary ductal carcinoma in situ but not in epithelial hyperplasia (20).

Role of Stem Cell Differentiation in Breast Carcinogenesis

Epidemiological studies indicate that nulliparous or late parous increased the risk for breast cancer (21, 22); lifetime lactation reduced the risk (22). This could be due to the induced mammary gland differentiation (23). Furthermore, from the atomic bomb studies in Japan, younger women with undifferentiated mammary glands were found to be at higher risk for radiation-induced breast cancer (24).

Recently, it has been shown that normal HBEC cultures lacking the CDKN2A (also known as $p16^{INK4a}$) expression can be immortalised by ectopic expression of the

human telomerase gene (TERT), whereas HBEC cultures expressing the CDKN2A failed to do so (25). Using a different culture system, we also found that HBEC cultures (Type II cells) derived from reduction mammoplasty vary in the expression of CDKN2A. In our experiments to extend the life span of HBEC by X rays, we found that one out of four independent cultures was deficient in the expression of p CDKN2A. Interestingly, this CDKN2A-deficient culture is the only one able to bypass senescence after repeated X irradiation (average cumulative population doubling of 24 clones = 32) (Table 1 and Figure 1). These extended-life (EL) clones eventually stopped proliferating. The TP53 and CDKN1A were frequently and concomitantly elevated in these EL clones in Western blot analysis. However, they appear to contain the wild-type *TP53* since the cells showed radiation-induced G_1-phase arrest (C. J. Albert Liu, MS Thesis, Michigan State University, 1997).

TABLE I
Induction of Extended Life-span (EL) Clones from Different HBEC Cultures by X Irradiation

HBEC culture	CDKN2A	No. of experiments	X-ray treatment[a] (dose × times)	Independent EL clones obtained
HME-5	+[b]	2	2 Gy × 4	None
HME12	+	2	2 Gy × 4	None
HME14	+	3	2 Gy × 3	None
HME14	+	2	4 Gy + 2 Gy × 3	None
HME15	−	1	2 Gy × 4	11
HME15	−	1	2 Gy × 5	5
HME15	−	1	2 Gy × 6	6
HME15	−	1	4 Gy × 5	2

[a]Approximately 5×10^6 Type II HBECs were used in the initial treatment with X rays.
[b]CDKN2A expression determined by Western blot analysis shown in Figure 1.

Figure 1. Expression of CDKN2A ($p16^{INK4a}$) in different normal HBEC cultures.

We have previously developed two types of normal HBEC from reduction mammoplasty (26). Type I HBECs, in contrast to Type II cells that expressed a basal epithelial phenotype, were deficient in gap junctional intercellular communication, expressed oestrogen receptors and luminal epithelial cell markers, and showed stem cell characteristics (i.e. the ability to differentiate into another cell type and to form budding/ductal structures in Matrigel) (26–27). Although both cell types can be equally transformed by SV40 large T-antigen to acquire extended life span, they differ in the ability to become immortal (11/11 Type I EL clones became immortal compared to 1/10 for Type II EL clones). Both parental Type I and Type II cells, as well as their transformed EL clones at early passages (~30 cpd), showed a low level of

telomerase activity measured by the TRAP assay. For all the 11 Type I and 1 Type II EL clones that became immortal, telomerase activities were invariably activated at middle (~60 cpd) or late passage (~100 cpd). For the senescent EL clones, the telomerase activities were found to be diminished at mid-passage before the end of lifespan. Thus, Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalisation, a mechanism making them major target cells for breast carcinogenesis (W. Sun, et al. Cancer Res., in press). We plan to use this cell culture system to test if Type I HBECs with stem cell features are also more susceptible to neoplastic transformation by ionising radiation.

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A Human Breast Epithelial Cell Type with Stem Cell Characteristics as Target Cells for Carcinogenesis

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Chang, C. C., Sun, W., Saitoh, M., Tai, M. H. and Trosko, J. E. A Human Breast Epithelial Cell Type with Stem Cell Characteristics as Target Cells for Carcinogenesis. *Radiat. Res.* 154, 000–000 (2000).

Two types of human breast epithelial cells (HBEC) have been characterized. In contrast to Type II HBEC, which express basal epithelial cell phenotypes, Type I HBEC are deficient in gap junctional intercellular communication and are capable of anchorage-independent growth and of expressing luminal epithelial cell markers, estrogen receptors, and stem cell characteristics (i.e. the ability to differentiate into other cell types and to form budding/ductal organoids on Matrigel). A comparative study of these two types of cells has revealed a high susceptibility of Type I HBEC to immortalization by SV40 large T antigen, although both types of cells are equally capable of acquiring an extended life span (bypassing senescence) after transfection with SV40. The immortalization was accompanied by elevation of a low level of telomerase activity in the parental cells after mid-passage (~60 cumulative population doubling levels). Thus HBEC do have a low level of telomerase activity, and Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalization, a mechanism which might qualify them as target cells for breast carcinogenesis. The immortalized Type I HBEC can be converted to highly tumorigenic cells by further treatment with X rays (2 Gy × 2) and transfection with a mutated *ERBB2* (also known as *NEU*) oncogene, resulting in the expression of $p185^{ERBB2}$ which is tyrosine phosphorylated.

INTRODUCTION

Studies of carcinogenesis have shown that neoplastic transformation occurs in progressive stages (*1*). The process involves multiple pathways and multiple mechanisms[2] (*2*), and a tumor arises as a result of the accumulation of a series of genetic and epigenetic alterations in several oncogenes and tumor suppressor genes (*1*). Recent studies of chemopreventive agents (*3, 4*) and of *in vitro* transformation using different cell types (*5–7*) further confirm the role of differentiation in neoplastic transformation. Therefore, the primary goal of the study of carcinogenesis is not only to identify oncogenes/tumor suppressor genes that are frequently mutated in tumorigenesis but also to identify target cells that are more susceptible to neoplastic transformation. In this respect, the advantage of using the *in vitro* neoplastic transformation model to study the mechanisms of carcinogenesis is the feasibility of stepwise analysis of specific genetic and molecular alterations during the course of neoplastic transformation and the unambiguous identification of the roles of different cell types in carcinogenesis.

THE ROLE OF STEM CELL DIFFERENTIATION IN CARCINOGENESIS

Cancer cells are generally recognized as being in a relatively undifferentiated state (*8*). This undifferentiated state of tumor cells could be due to either dedifferentiation of a differentiated cell or the blocked differentiation of stem cells that give rise to tumor cells (*9*). The latter view is similar to the earlier concept of cancer as a disease of cell differentiation (*10*) or of "oncogeny as blocked or partially blocked ontogeny" (*11*). Furthermore, several cancers (i.e. chronic myelogenous leukemia, B-cell non-Hodgkin's lymphoma, and multiple myeloma) are known to contain markers [e.g. the t (9:22) Philadelphia chromosome] that can be traced to hematopoietic stem cells (*12–14*). Thus these cancers have been described as a disease of stem cells (*12*). The considerably higher cancer risk for the large intestine relative to the small intestine might be ascribed to the expression of BCL2 in the stem cell position in crypts of the large intestine but not the small intestine (*15*).

In breast cancer, there is evidence that stem cell proliferation and differentiation play an important role in carcinogenesis. First, epidemiological studies indicate that the lifetime risk of breast cancer developing in child-bearing

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women is linearly related to the age at which a woman has her first full-term pregnancy (*16*) and that breast cancer risk is higher in those who are nulliparous or late parous (*17, 18*). This has been hypothesized as being due to stem cell multiplication that commences at the time of puberty and occurs during each ovarian cycle until, but not after, the first pregnancy (*19*). Alternatively and more likely, pregnancy might induce full differentiation of the mammary gland, resulting in refractoriness of the gland to carcinogenesis (*20*). In animal experiments, the protective effect of pregnancy against chemical carcinogen-induced mammary cancer can be mimicked by short-term postcarcinogen treatment with pregnancy levels of estradiol and progesterone in nulliparous rats (*21*). The results suggest that these hormones eliminate or modify preneoplastic cells. Similarly, human chorionic gonadotropin (hCG) (*22*) and genistein can induce mammary gland differentiation in rats and reduce chemical carcinogen-induced mammary carcinogenesis (*23*). Second, in the studies of the effects of radiation from the atomic bombs in Japan, the frequency of ionizing radiation-induced breast cancers has been found to be inversely correlated with the age of the women at the time of exposure, indicating a higher breast cancer risk for the young undifferentiated mammary gland (*24*).

In *in vitro* studies, different HBEC types have been shown to respond differently to human papilloma virus (*5*), X rays (*25*), and the catalytic component of telomerase, TERT (*6*), in life-span extension (*25*) or immortalization (*5, 6*). A major determinant of whether these cells are rendered more susceptible to tumorigenic initiation has been found to be the nonexpression of CDKN2A/RB (*6, 25*).

GENE MUTATIONS IN HUMAN BREAST CANCER

For breast cancer, several oncogenes (i.e. ERBB2, MYC) and G_1-phase cyclins (D and E) were frequently found to be amplified or overexpressed (*26–30*). Loss of heterozygosity was frequently observed on many chromosome arms in breast cancer (i.e., 1p, 1q, 3p, 11p, 13q, 16q, 17p, 17q and 18q) (*31, 32*). Some hereditary breast cancer genes are known to be located on these chromosome arms (i.e. *BRCA1,* 17q; *BRCA2,* 13q; Li-Fraumeni syndrome and *TP53,* 17p). The FHIT (fragile histidine triad) at 3p14.2 and wnt-5a at 3p14-p21 are candidate breast tumor suppressor genes on chromosome 3p (*33, 34*). The gene encoding a cyclin kinase inhibitor, CDKN1C (also known as $p57^{KIP2}$), whose overexpression causes G_1-phase arrest, was cloned and mapped to chromosome 11p15.5 (*35*). The remaining breast cancer genes on chromosome 1, 16q and 18q have not been positively identified. In addition, four tumor suppressor genes are likely to be involved in breast carcinogenesis: CDKN2A, a cyclin-dependent kinase inhibitor (*36*); the maspin, a gene encoding a protein related to the serpin family of protease inhibitors (*37*); the PTEN on chromosome 10q23, a protein tyrosine phosphatase (*38*); and the *ATM* gene, which is mutated in ataxia telangiectasia, a breast cancer-prone syndrome (*39, 40*) and whose products phosphorylated NFKB1A (also known as IKBA) (*41*) and TP53 (*42, 43*). Similar to the observations for *BRCA1* and *BRCA2,* the *ATM* gene has been ruled out as a frequently mutated tumor suppressor gene in sporadic breast carcinomas (*44*). Yet loss of heterozygosity of these three genes has frequently been found in sporadic invasive ductal breast carcinoma (*45*), suggesting the presence of other putative tumor suppressor genes in their vicinity.

Since cancer cells may arise from different pathways[2] and may involve different combinations of oncogenes and tumor suppressor genes, the exact interaction and interdependency of the above-mentioned and other oncogenes and tumor suppressor genes in breast carcinogenesis are not known. However, *TP53* inactivation appears to play a key role in tumor initiation. Besides its frequent mutations in breast cancer, the three known breast cancer-prone syndromes, Li-Fraumeni syndrome, ataxia telangiectasia (*46*) and BRCA1 (*47*), are known to affect the function or regulation of *TP53* or *CDKN1A* (also known as $p21^{WAF1/CIP1}$). This is corroborated by *in vitro* studies that show that transfections with dominant-negative mutant *TP53* were able to immortalize HBEC (*48–50*). However, *TP53* mutation may not be the very first event in breast cancer initiation since the mutation was found in mammary ductal carcinoma *in situ* but not in epithelial hyperplasia (*51*). Alternatively, *TP53* may be inactivated by epigenetic mechanisms and trigger tumor initiation.

Besides alterations in oncogenes and tumor suppressor genes, recent studies have shown that telomerase activation (or a telomerase-independent alternative telomere lengthening mechanism) also plays a key role in immortalization, a critical and perhaps rate-limiting step in the development of most human cancers (*52*). Indeed, telomerase activity was detected in more than 95% of advanced-stage breast cancers (*53*).

All together, despite the complexity of the mechanism, the major events in carcinogenesis appear to involve (1) altered cell cycle regulation—bypassing cellular senescence → (2) telomerase activation—immortalization → (3) activation of a growth-promoting pathway—tumorigenic → (4) altered cell adhesion, mobility and protease/collagenase activity—invasion and metastasis. After tumor initiation, the accumulation of mutations or epigenetic changes could be facilitated by mechanisms of tumor promotion (*54*) and genomic instability (*55*).

IN VITRO NEOPLASTIC TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

HBECs have been immortalized by various methods using benzo(a)pyrene (*56*), SV40 large T antigen (*57* and references therein), human papilloma virus 16 E6 or E7 (*5*), radiation (*58*), mutant *TP53* (*48–50*), and the catalytic component of telomerase, TERT (*6*). In these studies, cell type differences (e.g. early compared to late passage) signifi-

TABLE 1
Major Phenotypic Differences between Type I and Type II HBEC

	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobblestone appearance
Colony morphology	Boundary smooth and restricted	Boundary not smooth
Attachment on plastic surface after trypsinization	Late	Early
Effect of fetal bovine serum	Growth promotion	Growth inhibition
Gap junctional intercellular communication	Deficient	Efficient
Expression of:		
Connexin 26	−	+
Connexin 43	−	+
Epithelial membrane antigen	+	−
Cytokeratin 18	+	−
Cytokeratin 19	+	−
Cytokeratin 14	−	+
α6 Integrin	−	+
Estrogen receptor	+	−
Effect of cAMP (induced by cholera toxin, forskolin)	Induces Type I cells to change into Type II cells	
Organoid on Matrigel	Budding/ductal structure Acini	Spherical and elongated structures
Anchorage-independent growth	+ (small colony/low frequency)	−
Response to SV40 large T antigen:		
Anchorage-independent growth	+ (large colony/~~low~~ high frequency)	−
Immortalization	High frequency	Low frequency

cantly affect the outcome of experiments (*5*, *6*). ~~With the exception of~~ other than the viral oncogenes from SV40 or papilloma virus, the frequencies of immortalization by other agents are generally very low. With the exception of experiments using our Type I HBEC (*7*, *57*), none of the immortalized cell lines have been reported to express estrogen receptors and to be capable of anchorage-independent growth.

Spontaneously immortalized HBEC derived from fibrocystic mammary tissue or *in vitro* immortalized cell lines have been neoplastically transformed after benzo(a)pyrene treatment or *RAS* oncogene transfection (*58*–*61*). In our study, a Type I HBEC line immortalized by SV40 large T antigen and 5-bromodeoxyuridine/black light treatment was found to be tumorigenic in immune-deficient mice (*63*). The tumorigenicity of these cells can be further enhanced by the transfection with the *NEU* (now known as *ERBB2*) oncogene (*62*). In a different experiment, an SV40-immortalized cell line can be converted to weakly tumorigenic cells by X irradiation. The weakly tumorigenic cells became highly tumorigenic after transfection with a mutated ~~*ERBB2*~~ *NEU* oncogene (*63*). These highly tumorigenic cells expressed an elevated level of ~~ERBB2~~ that was tyrosine phosphorylated. In contrast, the ectopic expression of ~~ERBB2~~ *NEU* did not induce tumorigenicity in the immortal cell line. The ~~ERBB2~~ expressed in these cells was not tyrosine phosphorylated (*63*). p185 c-erbB2/neu

CHARACTERIZATION OF TWO TYPES OF HUMAN BREAST EPITHELIAL CELLS

We have previously developed a culture method for growing two morphologically distinguishable types of HBEC from reduction mammoplasty (*57*). These two types of cells are substantially different in many phenotypes (*7*, *57*, *62*, *64*) as listed in Table 1. The most significant characteristics of Type I HBEC that are related to breast carcinogenesis are (a) the expression of estrogen receptors; (b) stem cell features (i.e. ability of Type I cells to differentiate into Type II cells and to form budding/ductal organoids on Matrigel); and (c) the high susceptibility to neoplastic transformation by an oncogenic stimulus (i.e. SV40 large T-antigen-induced anchorage-independent growth and a high frequency of immortalization) (*7*, *57*).

Previously, we have found that Type I HBEC possess stem cell characteristics. The evidence came from the observation that Type I HBEC are deficient in gap junctional intercellular communication (GJIC) (*57*), similar to kidney and corneal stem cells (*65*, *66*). Furthermore, Type I HBEC were capable of differentiating into Type II cells by cyclic AMP-inducing agents (i.e. forskolin and cholera toxin) (*57*, *67*). The most convincing evidence, however, is the observation that Type I HBEC, in conjunction with Type II HBEC, form budding and ductal organoids in Matrigel (ref. *7* and Fig. 1) that are very similar to the human lobule Type I, as shown by Russo (*68*). These ductal structures can be formed overnight after the inoculation of a mixture of randomly distributed cells. After extended growth, lumen-like structures developed in these organoids. The Type II HBEC alone typically formed elongated and spherical organoids similar to the squamous metaplasia that developed in rat mammary cells in Matrigel (*69*).

Type I cells alone formed few budding (*7*) and acini structures similar to those formed by human luminal epithelial cells (*70*). Therefore, both luminal and stem cells are present in Type I HBEC cultures and are not morphologically distinguishable. Together with Type II HBEC, which

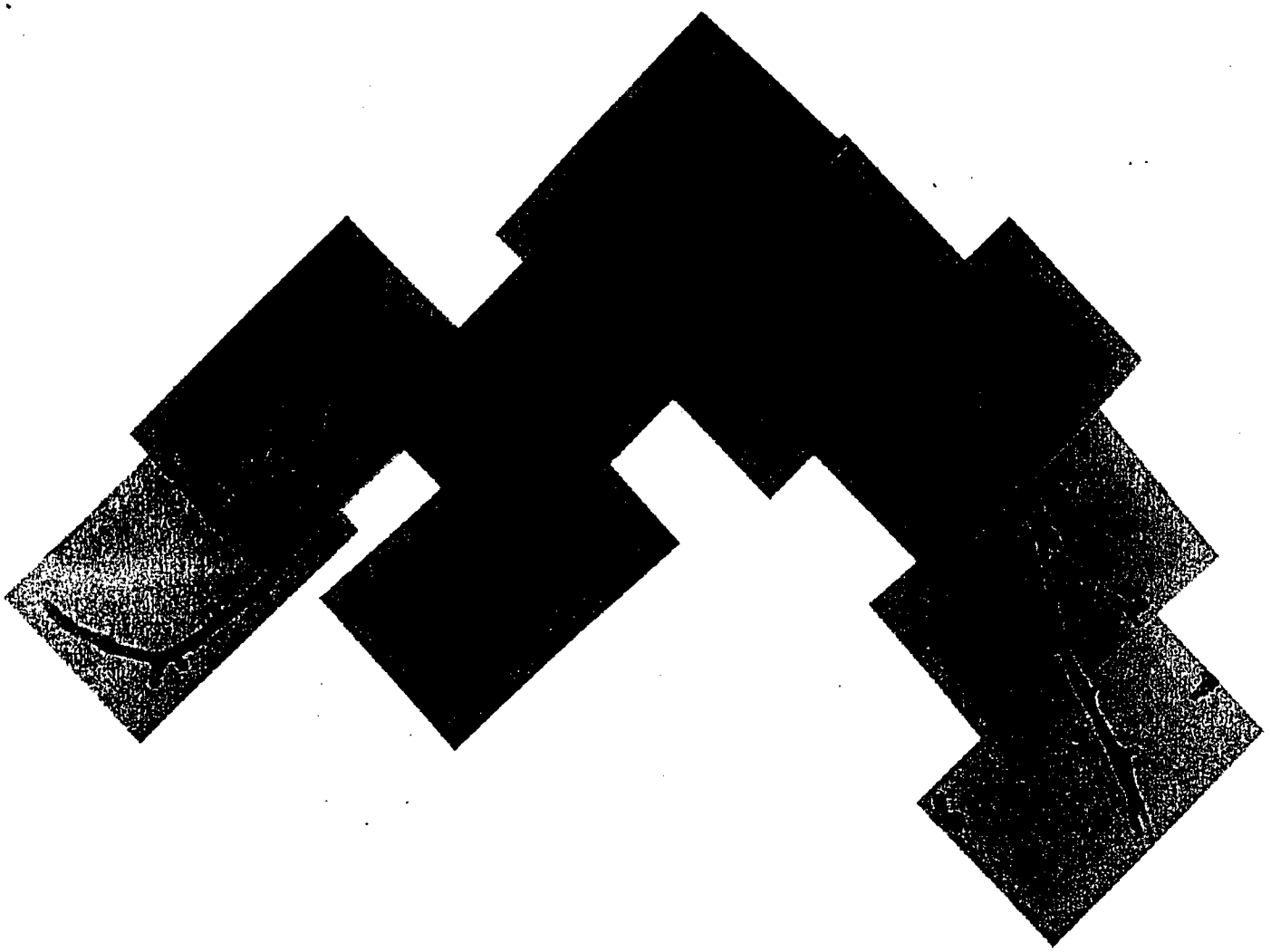

FIG. 1. Mammary organoid formed by mixing two types of human breast epithelial cells derived from reduction mammoplasty (Type I to Type II HBEC in 1:2 ratio) 1 day after inoculation of cells on Matrigel.

express basal epithelial cell phenotype, the three major breast epithelial cells in the mammary gland (stem, luminal, and basal or myoepithelial) are found in the two types of HBEC.

TYPE I HBEC WITH STEM CELL CHARACTERISTICS ARE MORE SUSCEPTIBLE TO TELOMERASE ACTIVATION AND IMMORTALIZATION

Since the two types of HBEC described above differ substantially in phenotypes and one of them shows stem cell characteristics, a comparative study was carried out to examine whether Type I HBEC are more susceptible to telomerase activation and immortalization after transfection with SV40 large T antigen (7). The results show that both types of cells acquire extended life span (i.e. bypassing senescence) at comparable frequencies. However, they differ significantly in their abilities to become immortal in continuous culture, i.e., 11 of 11 Type I extended life-span clones became immortal compared to 1 of 10 for Type II extended life-span clones. Both parental Type I and Type II cells as well as their transformed extended life-span clones at early passages (~30 cumulative population doubling levels) showed a low level of telomerase activity as measured by the TRAP assay. For all the 11 Type I and 1 Type II extended life-span clones that became immortal, telomerase activities were invariably activated at middle (~60 cumulative population doubling levels) or late passages (~100 cumulative population doubling levels). For the four Type II extended life-span clones selected randomly from the 9 Type II clones that did not become immortal, the telomerase activities were found to be diminished further at mid-passage before the end of their life span. Thus HBEC do have a low level of telomerase activity and Type I HBEC with stem cell characteristics are more susceptible to telomerase activation and immortalization, a potential mechanism to qualify these cells as major target cells for breast carcinogenesis.

The results of this study also provide some insight into and implications about breast carcinogenesis. First, unlike

previous reports that breast tissues (*71*) and HBEC (*6*) do not have telomerase activity, we found that both Type I and Type II HBEC do have a low level of telomerase activity, which needs to be elevated during immortalization. Second, a previous report concludes that telomerase activity may not be a biomarker for malignant transformation since it is present in both tumor and normal cells, including HBEC (*72*). This conclusion may be misleading since there was no quantitative comparison in that report and the comparison was made between tumor cells and unrelated normal cells. Using a well-characterized quantitative assay, we found that, although both primary breast epithelial cells and immortal or tumorigenic cells derived from them have telomerase activity, the activities in immortal and tumorigenic cells are significantly higher than that in primary cells. Third, the fact that SV40 large T antigen extends the life span (bypassing senescence) and telomerase activation effects immortalization further confirms that breast tumor initiation involves the tandem events of cell cycle deregulation and telomerase activation.

PERSPECTIVES

Our observations that Type I HBEC were more susceptible to telomerase activation and immortalization after transfection with SV40 large T antigen and that the resulting cells were capable of anchorage-independent growth and transformation to tumorigenic cells have implicated Type I cells as the major target cells for breast carcinogenesis. Furthermore, the similarity in phenotypes between Type I HBEC and breast carcinomas such as anchorage-independent growth, deficiency in GJIC, and expression of estrogen receptors and luminal epithelial cell markers (i.e. epithelial membrane antigen, cytokeratin 18, 19) supports the "oncogeny as blocked or partially blocked ontogeny" theory of carcinogenesis (*11*). However, our results based on transformation by SV40 need to be verified by experiments in which the cells are treated with other carcinogens. Recently, we found that co-transfection with a mutant *TP53* and the human *MYC* is a very effective method to extend the life span of HBEC. The resulting clones (some have more than 60 cumulative population doublings) express estrogen receptors and are capable of anchorage-independent growth. This method and other transformation methods will be used to test the hypothesis.

Although we have found the expression of variant and wild-type estrogen receptors in our Type I HBEC and in tumorigenic cell lines derived from them (*64*), the cell growth *in vitro* and tumor growth *in vivo* by these cells were not dependent on estrogen (our unpublished results). This may be because the cells had been transformed by SV40 large T antigen. Therefore, our goal is to obtain estrogen-responsive immortal cell lines by a different means of transformation. These cells will be very useful in other studies including testing the hypothesis that pregnancy levels of hormones (i.e. estradiol and progesterone) eliminate or modify premalignant mammary cells and mediate the protective effect of pregnancy against breast cancer (*21*).

The ability of the two types of HBEC to form mammary organoids in Matrigel similar to the human lobule Type I (*68*) suggests that these mammary organoids may be used as a surrogate for the human mammary gland to study the mechanisms of carcinogenesis as well as the regulation of mammary gland growth and differentiation in a more relevant cellular context. Furthermore, if Type I HBEC are target cells for breast carcinogenesis, they are also target cells for chemoprevention. Indeed, we have preliminary evidence that some potential chemopreventive agents such as genistein, vitamin D_3 and sphingosine are capable of inducing the differentiation of Type I HBEC into other cell types[3] (*73*). Therefore, Type I HBEC will be useful for studies of chemoprevention as well.

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Gap Junctions and the Regulation of Cellular Functions of Stem Cells during Development and Differentiation

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In multicellular organisms, the role of gap junction intercellular communication (GJIC) in the regulation of cell proliferation, cell differentiation, and apoptosis is becoming increasingly recognized as one of the major cellular functions from the start of the fertilized egg, through normal development of the embryo and fetus, to the sexual maturation of the adult and ultimately to the maintenance of health of the aging adult. Given that the function of this membrane-associated protein channel is to synchronize electrotonic or metabolic functions, differential regulation of function at the transcriptional, translational, and posttranslational levels of a family of highly evolutionarily conserved genes (connexins) needs to be considered. Both inherited mutations and environmental modulation of GJIC could, in principle, affect the function of gap junctions to control cell proliferation, cell differentiation, and apoptosis, thereby leading to a wide variety of pathologies. We review a few techniques used to characterize the ability of stem and progenitor cells to perform GJIC.

> Nothing in biology makes sense except in the light of evolution.
> T. Dobzhanski (1973, *Am. Biol.* **35**, 125–129)

This quotation by Dobzhanski (1) is being used to set the stage for the rationale to study a family of genes that code for the proteins called connexins. The cellular function of direct transfer of ions and small molecules between contiguous homologous or heterologous cells in a multicellular organism and the structural entity on the cell membrane, the gap junction, responsible for the means to connect the cytoplasms of these cells were only relatively recently discovered (2). With the transition of the single cell to the first multicellular organism came several important new phenotypic changes and the attendant genes responsible for these new functions.

Single-cell organisms survived by having all the intracellular signaling mechanisms to adapt to environmental changes and by unlimited proliferation (only physical factors such as temperature, pH, and external nutrients restricted cell proliferation). If the environment changed dramatically so as to cause death of most individual cells, the existence of a few spontaneous mutations in various genes might allow one of the population to survive to carry on the species. When the first multicellular organism appeared, the phenotypes of (a) the control of cell proliferation by "contact inhibition"; (b) the process of differentiation; (c) programmed cell death or apoptosis; and (d) the regulation of adaptive responses of terminal differentiated cells appeared (3). The process of "development" in a multicellular organism, therefore, must entail (a) the start from a single "totipotent" stem cell or fertilized egg (a cell that contains all the genetic material to form all the cell types and functions of the multicellular organism; a cell that has the ability to divide both symmetrically to expand its population and asymmetrically to have one daughter cell to terminally differentiate and the other to maintain "stemness"); and (b) the ability to selectively restrict the differentiation of this totipotent stem cell into a series of "pluripotent" stem cells (which can give rise to a restricted number of cell types for one

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or more tissues). These pluripotent stem cells can proliferate and differentiate into cells with even more restricted potentials to differentiate or proliferate (the progenitor cells of a given cell type or the terminally differentiated cells).

Of course, the transition from the single-cell organism to the first multicellular organism was accompanied by the appearance of many new genes. One new family, the connexin genes, which code for proteins needed for the formation of a hexameric hemichannel (the "connexon") to bond with the corresponding hemichannel in the contiguous cell membrane, appeared when the multicellular cellular organism appeared (4). Was it by chance, was it by coincidence, or was it causal that the connexin gene appeared when the control of cell proliferation, cell differentiation, programmed cell death, and adaptive responses of differentiated cells, processes needed for development, first appeared? Single cells could communicate with each other in a population by primitive molecules that could have been progenitors to hormones and growth factors (5). Single cells also had developed sophisticated intracellular signaling mechanisms that were the progenitors to many found in the multicellular organism. The delicate orchestration of cell growth, cell differentiation, and programmed cell death between the totipotent stem cells, pluripotent stem cells, their progenitors, and terminally differentiated daughters appears to have been mediated by the modulation of the genes that code for connexins that regulate gap junction function.

While at this stage, the aforementioned description of the transition from the single-cell organism to the multicellular organism is speculation, early attribution of the importance of gap junctions to the control of cell growth and differentiation (6), to adaptive functions of differentiated cells (7), and more recently to apoptosis (8, 9) has been made when one compares normal cells, which can have functional gap junction intercellular communication (GJIC), to that of cancer cells, which appear not to have either homologous or heterologous GJIC (10). It is interesting to note that cancer cells are characterized to be phenotypically unable to contact inhibit, to terminally differentiate, or to apoptose (11). Cancer has been described as a disease of differentiation (12), a stem cell disease (13), or as oncogeny as partially blocked ontogeny (14). In other words, if a stem cell is exposed to a carcinogenic agent that prevents it from completing its terminal differentiation program, it will be able to proliferate in an uncontrolled manner and is less sensitive to programmed cell death signals. In view of the demonstration that cancers appear to be monoclonally derived (15), can be induced to terminally differentiate or apoptose by certain chemotherapeutic agents (16, 17), and to have normal restored growth control and restricted tumor growth by agents that can induce GJIC or by transfection with connexin genes (11), the cancer cell, without functional GJIC, appears to be a throwback to evolution. The cancer cell behaves like a single-cell bacterium, having only intracellular signaling to respond to extracellular signals for proliferation, but none for growth control or differentiation.

Several important concepts appear to be emerging that demand explanations about the speculated roles that gap junctional intercellular communication plays during the transition of totipotent stem cells to the many pluripotent stem cells, progenitor and terminally differentiated cells as an organism goes through development. This process must allow stem cells to go from one cell to many (symmetrical cell division) and to differentiate while maintaining a stem cell pool for future growth and wound repair or tissue replacement (asymmetrical cell division). In addition, the mechanism allows a stem cell to maintain its stemness after its asymmetric cell division in the midst of its differentiated daughters. Consequently, isolation and characterization of human stem cells that are involved in development, differentiation, growth control, regulation of asymmetric/symmetric cell division, and apoptosis will be the objective of our recent work.

As a stimulus for our research into the role of stem cells in carcinogenesis, we needed to isolate human stem cells to test the hypothesis that stem cells are the target cells for carcinogenesis. Based on the observation that the totipotent stem cell or fertilized egg did not seem to express connexin genes or did not have the ability to perform GJIC (18), we assumed that all stem cells (toti- and pluripotent stem cells) maintained their primitive or undifferentiated state by being sequestered from their differentiated daughters. Two mechanisms seemed to provide this kind of sequestration: one being a physical barrier between the stem cell and its differentiated daughter; the other being the inability to couple via gap junctions with its differentiated daughter. While the lack of functional GJIC was observed in fertilized eggs and early embryos of *Xenopus, Drosophila,* and mouse (18), we assumed that in the tissues of most or all organs there existed many terminally differentiated cells and progenitor cells with functional GJIC. One other important observation suggested that the target cells for carcinogenesis did not have functional GJIC. In Syrian hamster embryonic cells, used to induce neoplastic transformants with chemical carcinogens, there exists a small population of contact-insensitive cells that were highly transformable by chemical carcinogens (19). Contact insensitivity appears to be the result of the lack of functional GJIC.

Lastly, in view of the potential of using human stem cells for tissue regeneration (20), the ability to isolate stem cells, based on their absence of GJIC, and to study the role of GJIC in the growth and differentiation of

stem cells is gaining importance. Fundamental questions that still must be answered are: (a) Is GJIC critical for growth control? (b) Is GJIC needed for cell differentiation? (c) Is GJIC necessary for apoptosis? (d) Do stem cells lack functional GJIC? Experiments and attendant techniques to answer these questions are described in this article.

DESCRIPTION OF METHODS

A. Techniques to Measure Gap Junction Function

A.1. The Scrape Loading/Dye Transfer Assay: Background

During development and differentiation, the alteration of GJIC occurs due to changes in the amount and type of connexin gene expression and posttranslational modifications of connexin proteins (21). Measurement of gap-junctional intercellular communication can be accomplished by many methods: metabolic cooperation, microinjection of single cells, fluorescence redistribution after photobleaching (FRAP), scrape loading dye transfer (SL/DT), and patch clamp to measure electrical conductance. Of all the techniques to measure GJIC, SL/DT is the fastest and simplest. This method was first described by El-Fouly *et al.* (22). Since then it has been used to assess the GJIC status of many cell types in various biological circumstances [for review, see (3)]. Since SL/DT can measure dye transfer in multiple cells almost simultaneously, it is the only visual method available to assess the overall GJIC of a population of cells (Fig. 1). We have used this assay to determine changes in GJIC after cell populations are transformed with oncogenes (Fig. 1B) or treated with a chemical tumor promoter (Figs. 1C, 1D) or a combination of chemical treatment of oncogene transformed cells (23).

A.2. Theory

Most gap junction channels exclude molecules greater than approximately 1000 Da. A dye such as Lucifer Yellow with a molecular mass of 457 Da can easily pass through gap junction channels. When a cell is loaded with such a dye, it will diffuse from one cell to another via gap junction channels if the cell is coupled with its neighboring cells as shown in Fig. 2A. Since Lucifer Yellow is brightly fluorescent (absorbance at 428 nm, emission at 536 nm), it is easily seen under a microscope using a UV light source. Other fluorescent dyes that are larger than the gap junction channel exclusion limit are employed as a control to label the initially loaded cell and show that dye transfer from cell to cell is not by other means such as cytoplasmic membrane fusions, cytoplasmic bridge formation, or leaky membranes that can occur under cytotoxic conditions. Rhodamine–dextran conjugates (10,000 MW, absorbance at 555 nm, emission at 580 nm) are most commonly used to label the primary (scraped) cells (Fig. 2B). The amount of dye transferred from one cell to its neighbor is dependent on the number of gap junction that are coupled and the gating properties of individual channels.

A.3. Materials

1. CaMg-PBS buffer: 137 mM NaCl (8g/liter), 2.68 mM KCl (0.2g/liter), 8.10 mM Na_2HPO_4 (1.15g/liter), 1.47 nM KH_2PO_4 (0.2g/liter), 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$. The PBS is sterilized by autoclaving. (Note: CaMg is added to the PBS buffer to prevent some monolayer cells from detaching from substrate. However, some cell types may be sensitive to this level of Ca^{2+}; therefore, by preparing the Lucifer yellow in CaMg-free buffer, one this problem might be alleviated).
2. Lucifer Yellow CH lithium salt (Molecular Probes, Eugene, OR), tetramethylrhodamine dextran (Molecular Probes).
3. 0.5 mg/ml Lucifer Yellow in CaMg PBS,
   0.5 mg/ml Lucifer Yellow and 0.5 mg/ml rhodamine–dextran in CaMg-PBS,
   0.5 mg/ml rhodamine–dextran in CaMg PBS. (Note: Up to 2 mg/ml rhodamine dextran may be used.)
   The solutions are filter-sterilized.
4. 4% (w/v) Formalin in CaMg-PBS.
5. Scalpel blades and Parafilm.

A.4. General Procedure

1. Cells are usually grown in 35- or 60-mm tissue culture dishes for 1 or 2 days.
2. The cells in question are rinsed multiple times with CaMg-PBS. The excess liquid may be removed with a pipet.
3. The Lucifer Yellow (with or without rhodamine–dextran) or rhodamine–dextran solution is added to the cell monolayer.
4. The end of a sterile scalpel blade is gently placed in contact with the cell monolayer and rolled in one direction to extend the contact with gentle pressure to wound cells in a straight line. Multiple scrapes should be performed on each monolayer. The plate is left undisturbed in dark or dim light for 3 min for complete dye transfer. The dye enters cells through wounds created by the scalpel blade and the wounded cell membrane heals in a few seconds. If cells contain functional gap junctions, the Lucifer Yellow dye travels through the gap junctions away from the cells along the scrape line into neighboring cells while rhodamine–dextran is retained in the initially loaded cells directly adjacent to the scrape line. The distance the Lucifer Yellow dye travels, within a designated period (usually a 3-min

period), away from the scrape line is indicative of the level of GJIC within a culture.

5. Cells are rinsed extensively with CaMg-PBS and fixed by adding 4% (v/v) formalin. The cells in PBS or medium can also be observed for GJIC without fixation.

6. Plates can be viewed immediately using an epifluorescence phase microscope with a UV light source and/or stored at 4°C for extended periods (months) sealed in Parafilm. Some workers have air-dried the fixed plates for extended storage and rehydrated them for viewing.

A.5. Quantitation of GJIC Using the SL/DT Assay

The distance the dye travels through a monolayer can be measured using a variety of methods: (1) two-dimensional determination of fluorescent area, (2) one-dimensional measurement with a ruler laid against a photograph, and (3) counting the number of fluorescently labeled cells. These methods are described below.

Fluorescent area. Typically we scan monolayers with the Ultima interactive laser cytometer (Meridian Instruments, Okemos, MI) to obtain digitized images. Any method used to obtain a digitized image of the scrape line and surrounding cells should be adequate. The fluorescence area, not intensity, is used to determine the extent of dye transfer within a monolayer. Many image analysis software programs have a subroutine for determining fluorescence area of a fluorescent image. The fluorescence areas of

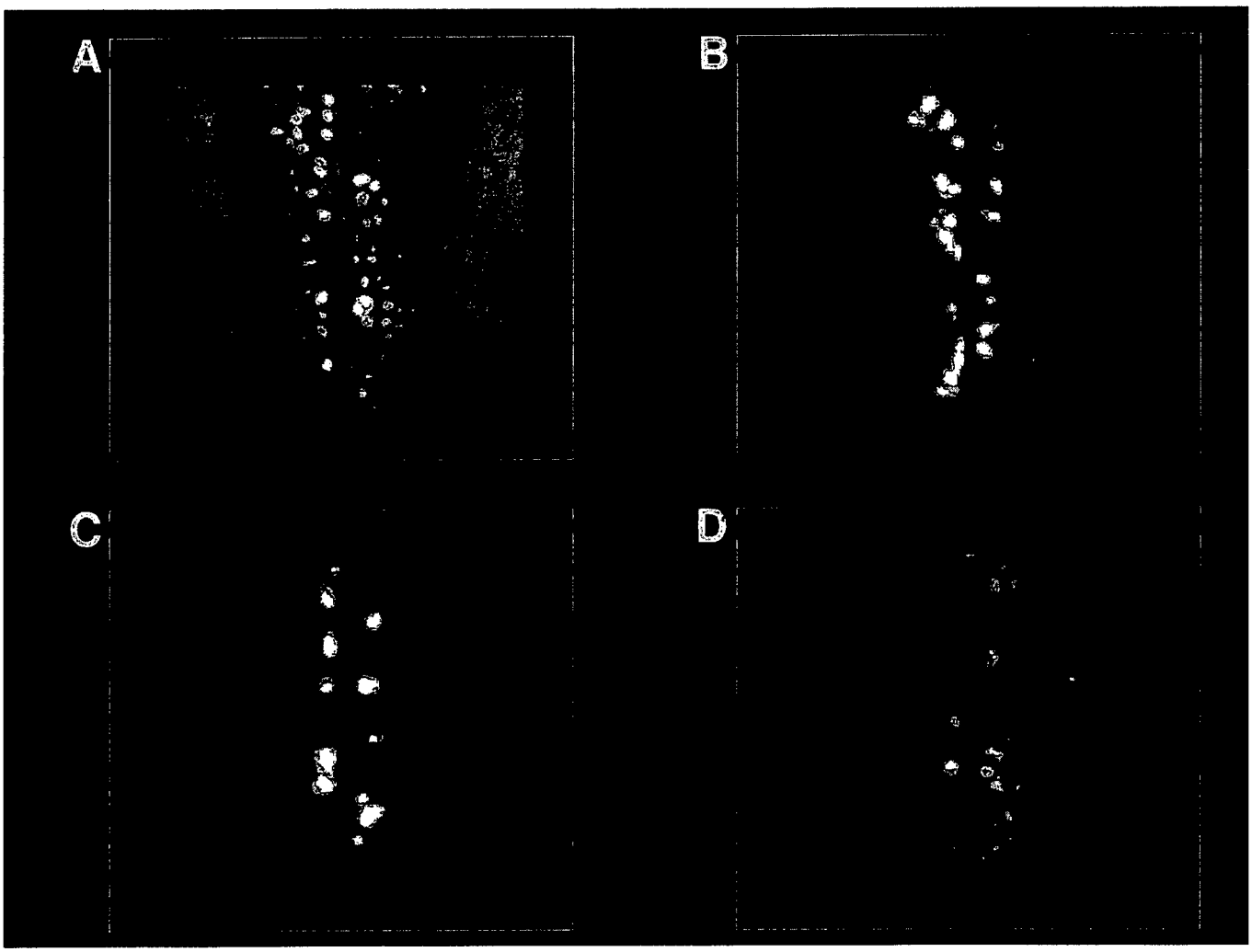


FIG. 1. Example of the scrape load/dye transfer (SL/DT) assay. Normal WB-F344 cultures (A), Ha-Ras transformed Wb-F344 cell line (B), and 30-min and 3-h TPA-treated WB-F344 (C and D, respectively) were subjected to the SL/DT assay as described in the text. Plates were scanned with a laser cytometer to obtain these digitized images. Spectral imaging was used to highlight the change in fluorescence intensity moving outward from the scrape line. White is the highest and pink is the lowest intenstity of fluorescence. Fluorescence area measurements were determined and percentage GJIC [as compared with normal WB-F344 (A)] was calculated as 17, 15, and 35% for (B), (C), and (D), respectively. Images are approximately equivalent to 200× magnification.

digitized images, which reflect extent of dye migration away from the scrape line, are subtracted from background fluorescence areas (areas of the monolayer well away from any scrape lines within the same test plate) to obtain a corrected fluorescence area value. These values can then be normalized to a control (test value divided by control) to obtain the fraction of control. Direct comparison of fluorescence areas between cultures is dependent on the consistency of the cells. All cells being compared must be relatively uniform with respect to growth state, cell type, and cell size. Generally, the average of 10 scrape line fluorescence areas is an adequate average to obtain a reasonable standard deviation for statistically significant data. A large standard deviation reflects a high degree of heterogeneity of growth,

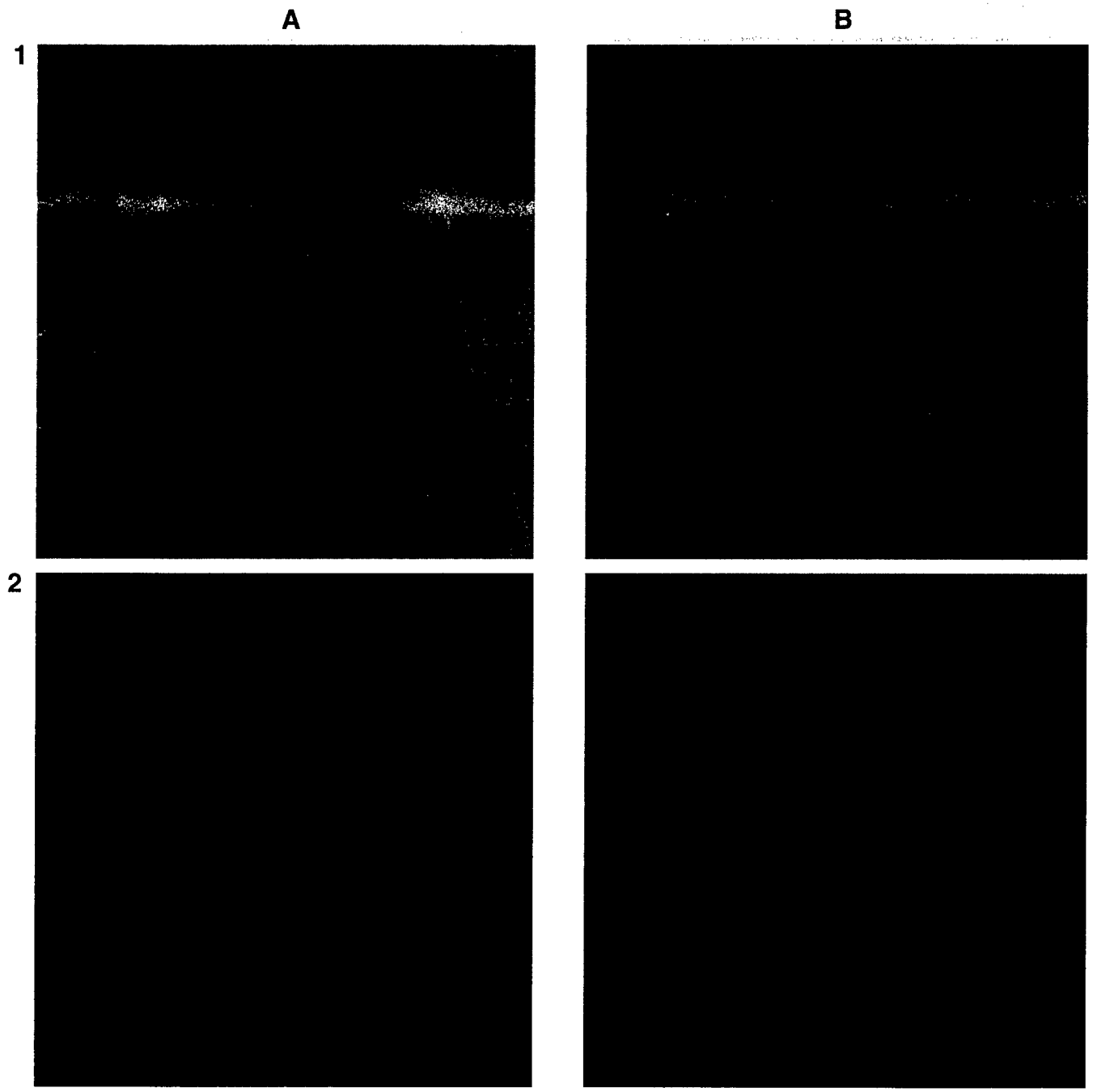


FIG. 2. SL/DT assay using a fluorescent dye excluded by gap junctions. WB-F344 cells were subjected to the SD/DT assay using Lucifer Yellow (A) and rhodamine conjugated to dextran (B). Phase-contrast (1) and UV transilluminated (2) photomicrographs were taken at 200× magnification. Lucifer Yellow was detected using a blue dichroic cube (410- to 485-nm exciter filter, 515- to 545-nm barrier filter) and rhodamine dextran was visualized using a green dichroic cube (535- to 550-nm exciter filter, 580-nm barrier filter).

size, and/or type of cells within a culture. If this is the case, a larger number of scrape line fluorescence areas should be included in the average.

One-dimensional measurement. If digitizing equipment is not readily available, or time consuming, measurement of distance traveled by the dye, using a standard ruler and photograph of the scrape lines, yields results similar to those obtained with fluorescence area measurements. However, this method can be highly subjective, since the experimenter decides extent of dye transfer. The distance the dye migrates is measured from the cell layer at the scrape to the edge of the dye front that is visually detectable. Due to the slight irregularity of the dye front edge, the distance along one scrape per dish is measured every 1 cm for a total of 8 cm (at 200×, 1 cm = 50 μm). The average cell length is 25 μm; therefore the 1-cm measurement on the photograph was equivalent to two cells. Each scrape chosen for photographic analysis is from a group of cells that are homogeneous in cell morphology and confluency. The nine measurements of distance are averaged and reported as the distance the dye traveled for the chosen group of cells representing the cell population of one monolayer. The photographs are taken and developed at the same time. Three plates per treatment are measured as described and the values are reported as an average ± 1SD.

Counting the number of fluorescently labeled cells. An alternative method of quantitating GJIC is by counting the number of cell rows adjacent to the scrape line that contain dye. There is a direct linear relationship between number of cell rows and level of fluorescence area if cell size, type, and growth state are similar (Fig. 3). Data calculations are similar to those described for the above two methods. This method may be useful when comparing populations of different cell size, type, and growth state.

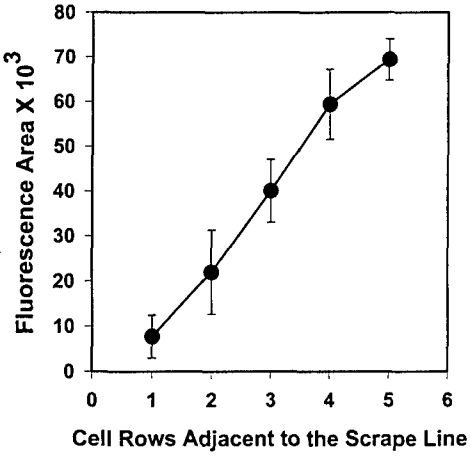


FIG. 3. Relationship between fluorescence area (ordinate) and number of cell rows (abscissa) Lucifer Yellow dye travels after cell monolayers are subjected to the SL/DT assay. Because the dye front can vary from one to two cells in a straight line, the data reflect n and $n \pm 1$ cells at each data point.

A.6. Data Analysis Considerations

When comparing different cell types or different treatment groups, it is absolutely essential that all cells compared are of uniform size. Generally we use analysis of variance for a test of significance for the results obtained by any of the methods listed above. Dunnett's multimean range t test is usually adequate for a test of significance between cell treatment groups and the control.

A.7. SL/DT: Drawbacks and Potential Misinterpretations of the Assay

The SL/DT is not useful for connexon channels that exclude molecules smaller than Lucifer Yellow. For example, connexin 45 channels cannot be assessed using Lucifer Yellow. If the connexin channels of a particular cell are unknown, then SL/DT using Lucifer Yellow as the transfer dye does not necessarily represent an accurate portrayal of the GJIC "competency" of that cell population. For channels that exclude Lucifer Yellow, smaller dyes such as biotin conjugates have been used (24).

The GJIC levels of some cell types of irregular shape are not easily quantitated using the SL/DT assay. For example, gap junction function of neuronal cells with extensive processes or long spindly fibroblasts cannot be easily quantified because the distance dye travels is not easily "trackable."

A.8. Fluorescence Recovery after Photobleaching (FRAP) in Cultured Cells: Background

The procedure described here was modified from one first reported (25) to measure GJIC between neighboring cells. In contrast to SL/DT, which measures GJIC in cell populations, FRAP is useful when studying individual cells within a population. This is extremely useful when assessing GJIC competency in cells displaying distinct morphologies such as associated with apoptosis and mitosis (9) or cells of neuronal origin (26) and fibroblasts in which GJIC is difficult to determine using SL/DT assay.

The general principle is to use a nontoxic, small-molecular-weight fluorescent dye that can pass through gap junctions in adjacent cells. Carboxyfluorescein diacetate (CFDA) was a suitable molecule. By selectively bleaching cells, the rate of transfer of fluorescent dye from the adjacent labeled cell back into the bleached cell could be calculated. In addition, it is possible to treat cells in a variety of ways that might affect their GJIC and document a corresponding effect on the fluorescent recovery rates.

A.8.a. Methods

Cell Labeling

Cultured cells are plated 1 to 2 days before the experiment is to be performed to allow for cell attachment, recovery from typsinization, and restoration of gap junctions. Cells should not be fully confluent since they need to be metabolically active to load with fluorescent dye and to communicate. However, there needs to be a significant number of touching cells for accurate measurements. For cultured human teratocarcinoma cells, if they were plated at a density of $1–2 \times 10^4$ cells per 35-mm dish, they would be suitable for experiments after 1 day of incubation.

Cells are incubated with CFDA (7 μg/ml; Molecular Probes, 1 mg/ml stock in ethanol) for 15–30 min at 37°C. In that form, CFDA is not fluorescent, but is permeable to the cells. An esterase enzyme function in the cells is necessary for the fluorescent molecule, carboxyfluorescein, to be released. There are some cell types with reduced esterase activity, and as mentioned above, senescent cells in confluence have extremely reduced levels and will not normally load sufficiently for accurate measurements. For maximum loading, a serum-free medium or isotonic buffer should be used (see recommended buffer described below). However, in experiments where serum was vital for parameters being measured, a reduced or normal serum concentration could be used. In these instances, increased CFDA concentration or incubation times may be necessary as the esterases in the serum often cleave a portion of the dye in solution, making it unavailable to the cells. After dye loading, the dishes were rinsed three times with buffer and kept in buffer or serum-free medium.

A.8.b. Photobleaching Measurements

The experiments described here were done using the Ultima (Meridian Instruments, Inc., now Genomic Solutions, Ann Arbor, MI). The computer-controlled instrument was equipped with an inverted microscope, argon laser tuned to 488 nm to excite the carboxyfluorescein, a microprocessor-controlled stage, and acoustoptic modulator to control beam intensity.

The dish of labeled cells was placed on the stage and a suitable field of cells was identified using a 40× objective. If desired, these experiments could be done with a stage system that maintains the culture dish at 37°C. For the optimum area, several touching cells should be located, along with one cell that is isolated from the rest which can serve as a control.

The field was scanned to generate a digital image of the fluorescence (Fig. 4, prebleach). Parameters of laser power, intensity used for scanning, photomultiplier setting, etc., were optimized to provide near-saturation levels in the image with minimal photobleaching. At this point, all the cells should uniformly be loaded with fluorescence. Often the nuclear region of the cell may appear brighter; this is typically a function of the cell being the thickest at that area and having the largest volume of fluorescent label.

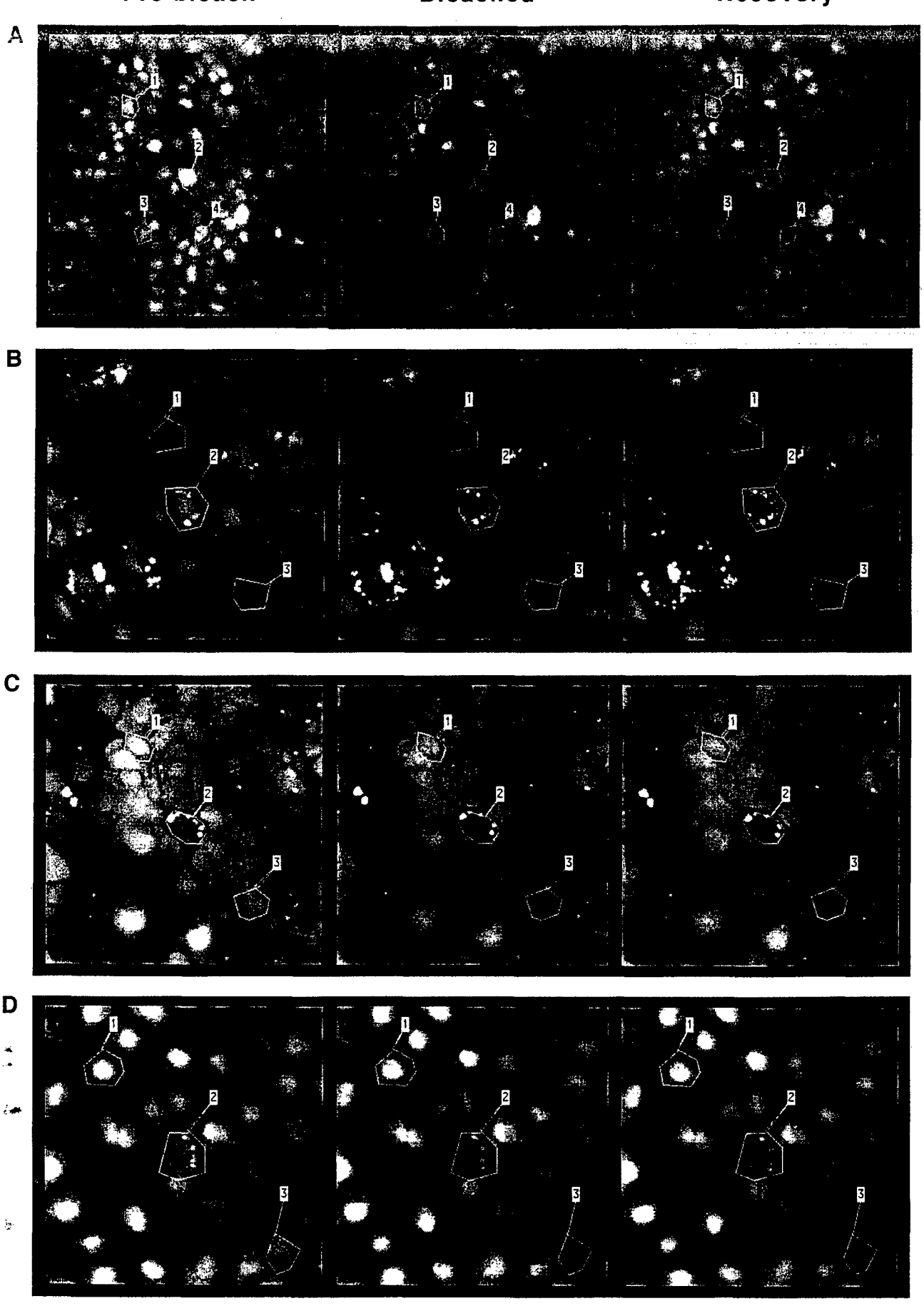
After the initial scan, selected cells were photobleached (Fig. 4A, bleached). With the Ultima, this is accomplished automatically with the software program by simply denoting which cells should be bleached with a more intense laser beam. Through use of an acoustooptic modulator, the intensity of the beam can be changed by orders of magnitude in microseconds, allowing scanning and bleaching to occur rapidly and sequentially.

After the bleaching has been accomplished, sequential scans to detect fluorescence recovery in bleached cells (Fig. 4A, recovery) are done at time intervals as determined by the experimenter. These images are digitally recorded for analyses. In addition to the adjuncent cells, several other controls should be done. These include an isolated cell that is bleached and should not recover because there are no adjacent cells from which dye can be transferred. Also several other nonbleached cells should be monitored to provide control data on the effect of several scans. Several fields can be rapidly scanned and bleached within one dish of cells to provide multiple data points.

A.8.c. FRAP Analysis

At the conclusion of the experimental portion of the experiment, the stored images can be recalled and the fluorescence quantified in the cells. This was accomplished by delineation of the cells to be monitored in the initial prebleached image that was stored. With the Ultima software, this same region was then monitored for fluorescence in all subsequent images and displayed as a percentage of the initial fluorescence recovery.

A typical plot of fluorescence recovery after photobleaching is shown in Fig. 5. The data have been normalized to the fluorescent signal present in each cell prior to photobleaching. In the cells that were bleached, the fluorescent signals drop to approximately 20% that of the prebleached levels, then gradually increase as fluorescent dye transfers into the cells through gap junctions. In an unbleached control cell, which was monitored for loss of fluorescent signal throughout the scanning process, the signal level remained constant at nearly 100% of the initial scan (Fig. 5, plot 1). In cells that were photobleached the positive slope of plots indicates recovery of fluorescence (Fig. 5, plots 2–4). In this example, cells 2, 3, and 4 showed rates of fluorescence recovery of 6.8, 12.7, and 13.0% per minute within the first 3 min of photobleaching. Additional detailed description of FRAP data analysis can be found in Stein *et al.* (27).


Pre-bleach
Bleached
Recovery
A
B
C
D

Buffer for Cell Loading and Experimentation

KCl	5.36 mM
NaCl	136.9 mM
KH_2PO_4	0.44 mM
$NaHCO_3$	4.16 mM
Na_2HPO_4 ($7H_20$)	0.336 mM
D-Glucose	11.101 mM
Hepes	5.0 mM
$CaCl_2 \cdot 2H_2O$	2.0 mM
BSA	0.1%

Adjust to pH 7.3; filter sterilize.

A.9. Heterologous GJIC: The FRAP Assay of Fluorescent Microbead-Labeled Cells: Background

Alteration of cell–cell communication between heterologous cell types is an important indicator of interactions that occur during various biological etiologies of differentiation, carcinogenesis, apoptosis, and mitosis (3). The difficulty in carrying out such experiments is in being able to distinguish between different cell types. For these experiments, one or both cell types are labeled with fluorescent microbeads; the cells are then mixed together in ratios such that one cell type is surrounded by another cell type (28). The FRAP assay is performed to determine the extent of GJIC of the cell type that is surrounded by the other cell type. This approach has been used successfully when assessing if GJIC has been altered after cells are transfected with chromosomes or plasmids or chemically/X-ray mutated (29–31).

A.9.a. Considerations

Although this is a relatively simple technique, many technical considerations must be addressed:

The bead concentration added to cells. If the concentration of beads in the medium is too high, then the cells tend to "gorge themselves" with beads and this affects the level of GJIC. If the bead concentration is too low, it is difficult to distinguish beaded from unbeaded cells.

The ratio of cells types mixed. Ideally the ratio is such that cells of one cell type completely surround a single bead-loaded cell of the other cell type and there are enough "heterologous clusters" to easily find fields to perform FRAP analysis. Typically a 1:25 ratio of beaded to unbeaded cells is adequate.

Cell confluency. The mixture of cells for each plate should be enough to form a monolayer of cells (90–95%) near confluency. Cells must be touching each other to form gap junctions.

Growth rate of cells. It is necessary to perform experiments within the first generation time after mixing labeled and unlabeled cell types. When a cell that contains microbeads divides, the beads do not necessarily segregate evenly to the daughter cells; thus a labeled cell can divide into an unlabeled cell and labeled cell, which leads to misidentification of the unlabeled daughter as the other cell type. A way to avoid this dilemma is to use beads labeled with two distinct dyes and label both cell types.

A.9.b. Materials

We typically use Polysciences, Inc., Fluoresbrite Carboxylate Microspheres, 1.16 μm in diameter. Molecular Probes is another good source of fluorescent beads of various sizes and fluorescent tags.

A.9.c. General Procedure

Labeling Cells with Fluorescent Beads

1. Grow cells in 35-mm cell culture dishes until they are 80% confluent.
2. Prepare beads: Dilute and wash the fluorescent bead stock by adding 1 drop of bead stock solution to 10 ml PBS. Microfuge 1 min at 2000 rpm. Discard PBS and resuspend the fluorescent beads in 10 ml of cell culture medium.
3. Remove medium from the cultures to be labeled. Transfer 2 ml of the bead suspension to each culture. Incubate overnight as per the cell growth conditions. The next day, rinse cultures extensively to remove any excess beads and check to see if the beads are evenly distributed within all cells in the culture. The concentration of beads in the medium and the confluency of cells used may have to be determined empirically for each particular cell type.

Preparing Cocultures

1. Trypsinize labeled and unlabeled cultures. Typically we add trypsin to a culture and remove it within

FIG. 4. Homologous and heterologous GJIC using the fluorescence redistribution after photobleaching (FRAP) assay. (A) Homologous GJIC WB-F344 cells were subjected to the FRAP assay as described in the text. Cell 1 remained unbleached throughout the assay as a control. Cells 2, 3, and 4 were bleached with 150-m sec pulses from a 488-nm argon laser. Fluorescence within these cells was monitored during the first 3 min after photobleaching and percentage fluorescence recovery was calculated to be 7, 13, and 13 %/min for cells 2, 3, and 4, respectively. Images depicted as 200× magnification equivalent. (B–D) Heterologous GJIC between Wb-F344 cells and WB.ab1, a mutant derivative of WB-F344 (60), was determined. Cells were labeled with fluorescent beads as described in the text, mixed (1:25 as WB*:WB (B), WB*:WB.ab1 (C) and WB.ab1*:Wb.ab1 (D) (where the asterisk denotes cells that were fluorescent bead labeled) and subjected to the FRAP assay. In all cases, cell 1 is the unbleached control, cell 2 is the fluorescent bead-labeled cell surrounded by unlabeled cell type, and cell 3 is usually an unbeaded cell. In (B), fluorescence recovery was 15%/ min in cells 2 and 3, whereas there was negligible fluorescence recovery in photobleached cells depicted in (C) and (D). Images depicted as 400× magnification equivalent.

30 s, then allow the cell to become detached from the plate.

2. Add 2.0 ml of cell culture medium to each culture; gently pipet up and down.

3. Transfer 80 μl of trypsinized fluorescent bead-labeled cells to a trypsinized culture of unlabeled cells containing 2.0 ml of trypsinized cells. Mix by gently pipetting up and down. If both cultures are 80–100% confluent, then the ratio of beaded to unbeaded cells in the coculture is approximately 1:25. Incubate overnight in medium containing reduced serum (1%) to minimize cell division.

FRAP Analysis

1. FRAP analysis can be performed essentially as described in Section A.8.

2. To obtain significant data we typically subject approximately 50 cells per coculture group to FRAP analysis.

3. Controls: For each coculture pair, homologous and heterologous bead controls must be included in all experiments. For example, if we want to determine if cell type A will form gap junctions with cell type B, the following coculture groups should be assessed by FRAP (an asterisk denotes fluorescent bead-labeled cells):

A*/A B*/B
A*/B A/B*

Data are expressed as the rate of fluorescence recovery per minute. Examples of typical data are described in Figs. 4B, 4C, and 4D.

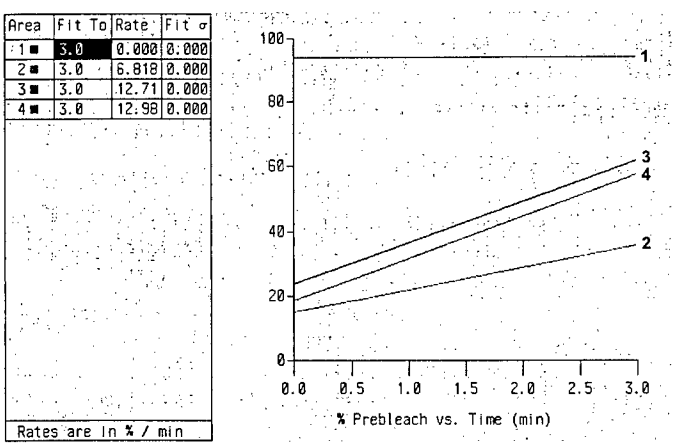


FIG. 5. Fluorescence recovery plot of a FRAP experiment. Data are for the experiment depicted in Fig. 4A. Cell 1 (unbleached control) is used to correct for entire field photobleaching due to multiple laser scanning of each field. Percentage prebleach fluorescence (ordinate) was plotted against time, in minutes, after photobleaching (abscissa).

A.10. Regulation of Human/Rat Connexin Transcription in Cells: Quantitation of mRNA

A.10.a. Expression of mRNA Transcripts Measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR): Background

Amplification of individual RNA molecules can be achieved by a method that combines reverse transcription (RT) and polymerase chain reaction (PCR). This method has been demonstrated to be extremely sensitive for mRNA analysis, and semiquantitative information can be obtained. Therefore, this technique is a powerful tool to study the regulation of transcription. In the PCR step, the mRNA of interest is specifically amplified by using two gene-specific oligonucleotide primers that anneal in two different exons to discriminate between genomic DNA. Relative RNA levels can be measured by relating the amplicons derived from the mRNA of interest to those of a housekeeping gene. The latter functions as control because its transcription is not affected by inducers.

A.10.b. Materials

RNA Preparation

Total RNA was extracted from cells by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions (32). Briefly, cells (2–5 × 10^6 cells) were washed twice with PBS, lysed by adding 1 ml of TRI Reagent, and transferred to microcentrifuge tubes. The samples were then incubated for 5 min at room temperature, 0.2 ml of chloroform was added with vigorous shaking for 15 s, and the mixtures were incubated at room temperature for another 10 min and centrifuged at 12,000 rpm for 15 min at 4°C. Each aqueous phase was transferred to a fresh tube, and the RNA was precipitated by mixing with 0.5 ml of 2-propanol. The samples were then incubated at room temperature for 5–10 min and centrifuged at 12,000 rpm for another 10 min at 4°C. The gel-like RNA pellet was washed with 75% ethanol by vortexing, centrifuged at 12,000 rpm for 10 min at 4°C, and air-dried for 10 min. The extracted RNA was dissolved in $T_{10}E_1$ with a RNase-free DNase cocktail containing (per sample) 10 μl of 100 mM $MgCl_2$/10 mM dithiothreitol (DTT) solution, 0.2 μl of 2.5 mg/ml RNase-free DNase, 0.1 μl placental ribonuclease inhibitor, and 39.7 μl TE buffer. The samples were mixed and incubated for 15 min at 37°C. The DNase reaction was stopped by adding 25 μl DNase stop mix containing 50 mM EDTA, 1.5 M sodium acetate, and 1% sodium dodecyl sulfate (SDS). The RNA was extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The extracted RNA was precipitated by adding the same volume of ethanol and centrifuged at 12,000 rpm for 10 min at 4°C. The RNA pellet was washed with 75% ethanol by vortexing,

centrifuged at 12,000 rpm for 10 min at 4°C, and air-dried for 10 min. Finally, the extracted RNA was dissolved in RNase-free water, and its RNA concentration was determined by spectrophotometry at 260 nm.

A.10.c. General Procedure

Reverse Transcription

It is advantageous to prepare total cDNA as PCR template, through the use of an oligo(dT) primer for the RT reaction. This alternative to using a gene-specific RT primer ensures an opportunity to reuse each sample multiple times and to reserve samples for future analysis for the expression of additional mRNAs. Reverse transcription of 1 μg of total RNA was performed in a final volume of 20 μl for 1 h at 37°C, using 50 U of M-MLV reverse transcriptase (Strata Script RNase H^- reverse transcriptase, Stratagene, La Jolla, CA) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 25 U of RNase inhibitor, 1 mM each of dATP, dGTP, dCTP, and dTTP, and 2.5 mM oligo(dT) (Pharmacia AB, Uppsala, Sweden). The samples were then heated to 90°C for 10 min to terminate the RT reaction, and quick-chilled on ice.

Oligonucleotides Used for PCR Amplification

Oligonucleotides were synthesized on an Applied Biosystems automated synthesizer. The primers for the PCR amplification of human or rat Cx26, Cx32, and Cx43 are RNA-specific primers. The 5′ primers are located on the first exon, and the 3′ primers are located on the second exon. The sequences are listed in Table 1.

PCR Amplification

The reverse-transcribed cDNA obtained from total RNA was added to a reaction mixture that contained a final concentration of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 mM each primer of Cx26, Cx32, or Cx43, 0.5 mM each primer of GAPDH, as a control for integrity of RNA, and 2.5 U of *Taq* polymerase (Perkin-Elmer–Cetus, Norwalk, CT) in a final reaction volume of 20 μl. The mixture was heated at 95°C for 30 s in a Perkin-Elmer DNA thermocycler (Perkin-Elmer–Cetus). Amplification was performed in 15 to 35 sequential cycles at 95°C for 20 s, 58°C for 40 s, and 72°C for 40 s, followed by incubation for 7 min at 72°C. The PCR products were analyzed on a 8% polyacrylamide gel in 1× Tris–borate/EDTA buffer. Gels were stained with ethidium bromide and photographed using Polaroid Type 667 film.

Optimal Conditions of RT-PCR

Each PCR assay needs to be optimized in several respects. First, specific amplification of the sequence of interest must be ensured. This step involves initially determining that PCR amplification yields discrete products of the expected size on ethidium bromide-stained polyacrylamide gels. Next, PCRs are saturable, and distinction between inputs is lost at cycle numbers that over- or underamplify. Analysis of products from reactions that have not been optimized yields large errors in estimation of target mRNA concentration. Such errors usually bias toward underestimation of differences between samples. Cycle number titrations are conveniently performed by scaling PCRs to 100 μl and removing 10-μl aliquots at each step. Preparation of parallel reactions that are subjected to varying cycle numbers is an adequate alternative.

As an example, two kinds of cell lines, G401.2/6TG.1 epithelial cell line (G401.2/6TG.1) and WB-F344 cell line (WB), were used to determine optimal PCR cycle number. The G401.2/6TG.1 epithelial cell line, derived from the kidney of a Wilms' tumor patient, exhibited extensive GJIC (33). The Fisher 344 rat liver-derived epithelial cell line, WB-F344, was obtained from J. W. Grisham (University of North Carolina) (34). The relationship between cycle number and PCR product was determined with these cell lines. The kinetics of RT-PCR amplification of human and rat GAPDH and Cx43

TABLE 1

Oligonucleotides of 5′ Primers and 3′ Primers of Connexin Genes and GAPDH Genes

mRNA species	5′ Primer	3′ Primer	PCR product (bp)
Human			
Cx26	GATTTAATCCATGACAAACT	CCACACCTCCTTTGCAGC	558
Cx32	GGGAAAGGGCAGCAGGAG	CCCATGGCCCTCAAGCCG	627
Cx43	GCGTGAGGAAAGTACCAAAC	GGGCAACCTTGAGTTCTTCC	506
GAPDH	GTTCGACAGTCAGCCGCATC	GTGGGTGTCGCTGTTGAAGTC	933
Rat			
Cx26	CGCGGCCGTCCGCTCTCCCAA	GAAGTAGTGGTCGTAGCACAC	481
Cx32	AGGTGTCGCAGTGCCAGGGAG	CCCGTGCCCCTCAAGCCGTAG	373
Cx43	GCGTGAGGAAAGTACCAAAC	GTGAAGCCGCCGCCAAAGTTG	527
GAPDH	TGAAGGTCGGTGTCAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	983

genes in the G401.2/6TG.1 and WB-F344 cell lines are shown in Figs. 6A and 6B and Fig. 7A and 7B, respectively. The A panels are densitometric quantitations of each band in the gel shown in the B panels. RT products are subjected to cycle number titration, typically in two stages. Initially, cycles are increased in increments of 5 from 15 to 35 cycles, producing a broad cycle number titration for this primer pair. The relationship between cycle number and PCR product is then determined. An optimal cycle number is determined at a titration across the exponential portion of the curve, using increments of a cycle at each step; the cycle number that represents the midexponential portion of the curve is selected. The cycle number that is defined in this fashion is then validated by cDNA input titration, using serial dilution of RT reaction product to program PCR amplifications, at the established optimal cycle number. Cycle number optima that are practically useful will yield outputs that are linear with respect to input across 2 logs of serial cDNA input dilutions. The successful application of a predetermined optimal PCR cycle number relies on the assumption that varying levels of mRNA starting material will produce RT reaction products that directly correspond to input. This assumption is directly tested for each assay by RNA input titrations into the optimized assay. The output should remain linear across at least 1 log of input.

Assessment of Cx mRNA Expression

As an example, the Cx mRNA levels of two cell lines, G401.2/6TG.1 and WB, were examined. As shown in Fig. 8, these cell lines were shown to express both Cx26 and Cx43 gap junction proteins. The mRNA of human/rat Cx26 and Cx43 was amplified and detected by RT-PCR. On the other hand, rat hepatocytes were shown to express both Cx26 and Cx32 gap junction proteins and the mRNA of rat Cx26 and Cx32 was amplified. These results confirm the previous results obtained from Western blot analysis and immunostaining analysis with specific antibodies against Cx26, Cx32, and Cx43 proteins.

A.10.d. Comment on RT-PCR Analysis

Quantitative PCR methods for comparing the relative amounts of mRNA in different cells have been reported (35, 36) and the quantitation has since been improved by competitive PCR, the coamplification of a known amount of an allelic gene variant as an internal

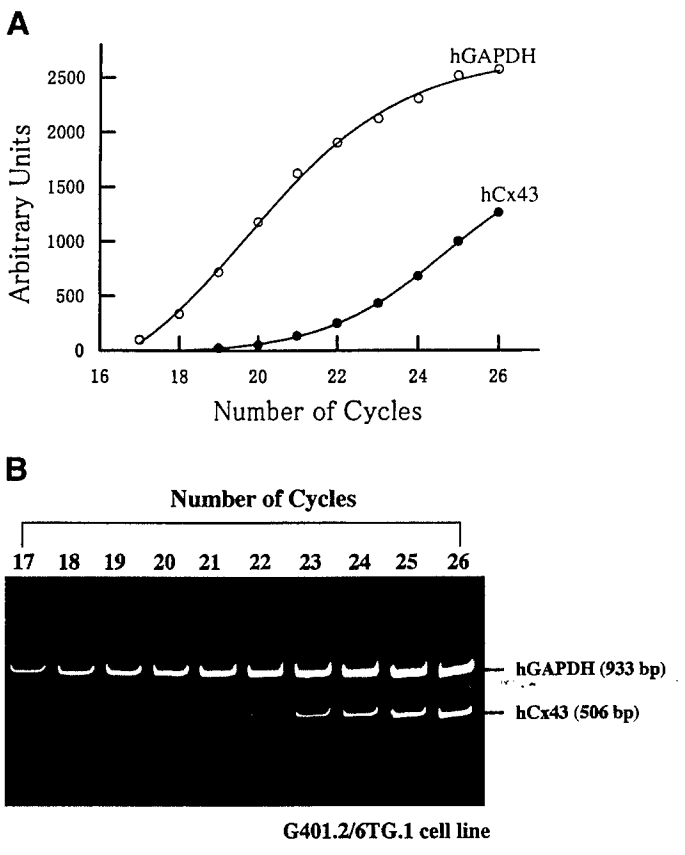


FIG. 6. Densitometric quantitation (A) of each band reflecting the GAPDH and connexin 43 genes in the gel shown in (B) of human G401.2/6TG.1 cell line.

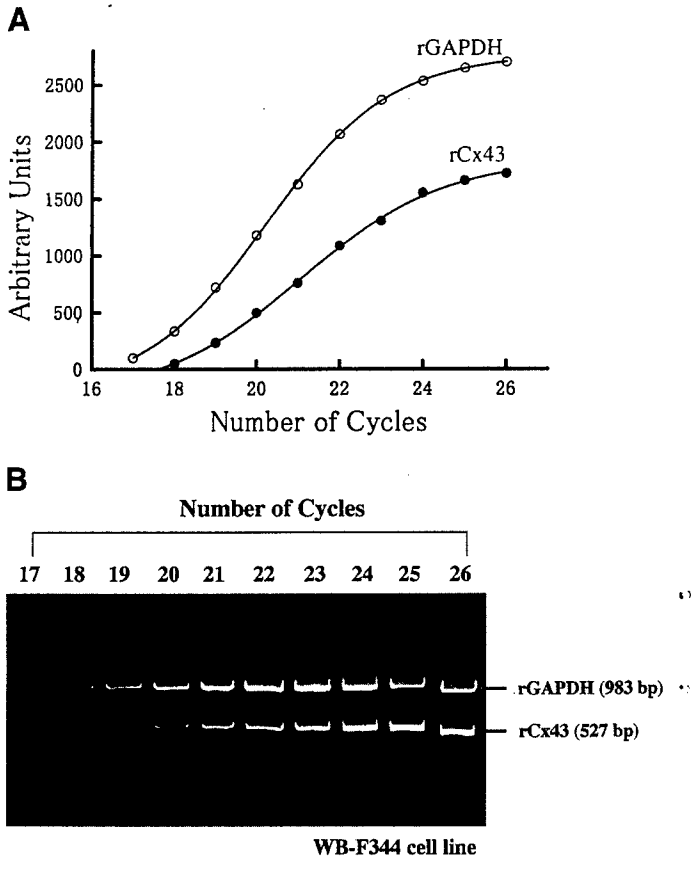


FIG. 7. Densitometric quantitation (A) of each band reflecting the GAPDH and connexin 43 genes in the gel shown in (B) of rat liver epithelial (WB-F344) cells.

standard (37, 38). These methods, however, are limited to measuring the mRNA level of a single gene. To measure the expression of more than one gene, an internal standard with the same sequences as the primers could be constructed and coamplified, but this would be costly and impractical. The multiple PCR has been shown to be a reliable method for the simultaneous amplification of two genes, and the results from this technique are in good agreement with the results of competitive PCR amplification of a target cDNA and an internal competitive template (39). This method is a nonradioactive multiplex RT-PCR technique for the rapid simultaneous amplification of two genes and quantitation of the relative levels of three kinds of connexins. Development of a new assay requires approximately 1 week.

A.11. Western Blot Analysis of Connexins

A.11.a. Background

Intercellular communication through gap junctions plays a critical role in maintaining the homeostasis of an organism by controlling the epigenetic expression of genes that establish a balance between cell proliferation, apoptosis, differentiation, and adaptive responses to environmental stimuli (3). Gap junction proteins form channels between contiguous cells, allowing the transfer of small signaling molecules up to a molecular mass of 1200 (40). A family of highly conserved genes codes for the gap junction proteins of which 13 mammalian gap junctions have been discovered thus far (3). Gap junctions in different tissues are not identical but they do share a basic structure. The gap junction consists of six protein subunits called connexins, which form a hexameric channel called a connexon. The connexon of one cell docks with the connexon of an adjacent cell to form a continuous channel between the two cells, thus forming a gap junction. However, the control of GJIC depends on the type of connexins that form the channels in the various types of cells found in each tissue. The connexon can be a homotypic–hexameric channel consisting of only one type of connexin, but a heterotypic channel consisting of two or more different types of connexins has been hypothesized since most cells express more than one type of connexin (41). Therefore, our understanding of the mechanism by which gap junctions are regulated must first depend on determining which connexins exist in the cell type being studied.

The most common method used in determining the type and size of connexins found in a cell is Western blot analysis. Western blot analysis of proteins is a well-established technique that entails electrophoretic separation of proteins in a gel matrix followed by electrophoretic transfer of the proteins from a gel to a paper matrix. The paper matrix is much more conducive to enzyme-linked immunosorbent assays than the gel. Specific antibodies can therefore be used to identify the particular connexins found in a given cell type. Western blot analysis can also be used to detect post-translational changes such as the phosphorylation status of the connexins.

A.11.b. Methods

Protein Extraction

Cells are normally grown between 90 and 100% confluency. Extractions are usually easier from cells grown in tissue culture plates than flasks. Usually 1×10^6 cells yield an adequate amount of protein and can be easily attained using 35-mm-diameter cell culture plates. Cells are rinsed three times with PBS and the plates are tipped on a paper towel to drain off all excess liquid. The lysis buffer (125 μl/10 cm^2) containing 20% SDS and 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) is added and the plates are shaken to evenly coat the cells with the lysis buffer. The cells are then scraped with a disposable cell scraper (Sarstedt, Newton, NC) and transferred to a 500-μl microcentrifuge tube and sonicated with three 10-s pulses at 35% maximal power using an ultrasonic probe to sheer the DNA. To avoid protein degradation, the samples are frozen and thawed only once for each Western blot analysis and protein determination. Therefore, the sample should be separated into several aliquots before freezing at −20°C. Also, to minimize the effects of phosphatases on the dephosphorylation of connexins, we have added 100 μM Na_3VO_4 to the PBS washes, and 100 nM aprotinin, 1.0 μM leupeptin, 1.0 μM antipain, 100 μM Na_3VO_4 and 5.0 mM NaF to the lysis buffer. Addition of these phosphatase inhibitors is not always

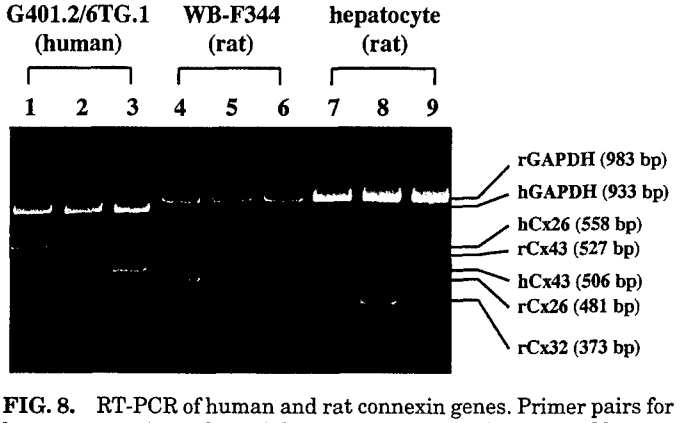


FIG. 8. RT-PCR of human and rat connexin genes. Primer pairs for human connexin 26 (lane 1), human connexin 32 (lane 2), and human connexin 43 (lane 3) were used to amplify RNA isolated from G401.2/6TG.1 cells. Primer pairs for rat connexin 26 (lanes 4, 7), rat connexin 32 (lanes 5, 8), and rat connexin 43 (lanes 6, 9) were used to amplify RNA isolated from rat liver oval cells (WB-F344) and primary hepatocytes. GAPDH was coamplified as a control. Expected PCR product sizes are indicated to the right.

necessary because comparative experiments in control and TPA-treated WB-F344 rat liver epithelial cells showed no difference between these two extraction procedures (data not shown). However, these results do not rule out the possibility that phosphatases could artifactually dephosphorylate connexins in other cell types.

SDS–PAGE

The proteins can be separated using discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (42). There are several commercially available gel boxes of varying sizes available. We use the Mini-PROTEAN system from Bio-Rad (Hercules, CA). A 12.5% acrylamide gel is used to separate the various isoforms of connexins. Electrophoresis-grade reagents should be used for all solutions. The separating gel consists of 12.5% acrylamide, 0.33% methylbisacrylamide, 0.1% SDS, 0.375 M Tris/pH 8.8, 0.05% ammonium persulfate, 0.05% *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and the stacking gel contains 4.0% acrylamide, 0.1% methylbisacrylamide, 0.1% SDS, 0.125 M Tris/pH 6.8, 0.05% ammonium persulfate, 0.10% TEMED. The running buffer contains 0.025 M Tris/pH 8.4, 0.1875 M glycine, 0.1% SDS. An adequate amount of protein for loading onto the gels is 15–20 μg. The protein samples are first mixed with an equal volume of 2× sample buffer containing 20% glycerol, 1.4 M 2-mercaptoethanol, 4% SDS, 0.125 M Tris/pH 6.8, 0.2% bromphenol blue. Unlike sample preparations for most SDS–PAGE runs, connexin samples are not boiled or heated to 60°C. Rainbow (Amersham International, Arlington Heights, IL) colored protein molecular weight markers are a convenient way of not only determining approximate molecular masses of the protein bands but also monitoring the electrophoretic run when there is a need to run beyond the bromphenol blue dye front. For connexin 43, gels are normally run at a constant potential of 200 V for 1 h 20 min, which is when the pink MW marker (lysozyme, 14.3 kDa) reaches the bottom of the gel. This will achieve the maximal resolution of the three connexin 43 bands.

Electrophoretic Transfer

After the electrophoretic run, the stacking gel is cut off and discarded and the separation gel is removed from the plates and soaked in transfer buffer for 10 min with no agitation. If PVDF transfer membranes (Millipore Corp., Bedford, MA) are used, membranes need to be soaked in 100% methanol for 60 s and then rinsed for 5 min in distilled water. The assembly of the gel transfer membrane sandwich consists of layering first the mesh pad followed by blotting paper, gel, transfer membrane, blotting paper, and mesh pad. To ensure good contact between the transfer membrane and gel and removal of air bubbles, a makeshift rolling pin should be passed over the sandwich with firm pressure before the last mesh pad is put on. All components should be premoistened and the transfer buffer (0.025 M Tris/pH 8.4, 0.1875 M glycine, 0.02% SDS, 20% methanol) should be chilled to 5°C. The sandwich must be placed in the transfer tank with the gel facing the anode side and the transfer membrane facing the cathode side. We normally run the transfer for 15–20 h at a constant potential of 20 V and use one to two ice packs throughout the run. Alternatively, shorter transfer times can be achieved with higher voltages.

Immunoblotting

The transfer membrane is removed from the sandwich and rinse once with distilled water. Ponceau protein staining dye is added to the transfer membrane (Sigma Chemical Co., St Louis, MO) for 30–60 s and then the membrane is rinsed with distilled water until there is a good contrast between the background and protein bands. The Ponceau-stained membrane can be recorded either by a photograph, benchtop scanner, or gel doc system. The latter two methods are more conducive for objective data analysis using computer software that can quantify densiometric scans. These data serve as a background measurement of total protein. After Ponceau staining, the membrane is treated with a blocking reagent for 1 h under gentle agitation. A suitable and economic blocking reagent is 5% Carnation nonfat dried milk dissolved in PBS + 0.1% Tween (PBS/Tween) that has been filtered through Whatman No. 1 filter paper. The primary and secondary antibodies are also dissolved in the PBS/Tween buffer. When blocking is complete, the primary antibody is added for 2–24 h under gentle agitation at a dilution of 1:500 to 1:3000. Treatment times and dilutions are highly dependent on the amount of connexin of interest present in the cell. For Cx43 in WB cells, a 1:2000 dilution of polyclonal anti-Cx43 (Zymed Inc., South San Francisco, CA) for 2 h is sufficient for good detection. Before the secondary antibody is added, the membranes are thoroughly washed to remove excess/unbound antibody. The wash consists of one 15-min rinse followed by two more 5-min rinses using fresh PBS/Tween for each wash. Following the washes a 1-h treatment under gentle agitation with a 1:1000 dilution of the secondary antibody is normally sufficient for good detection. Again the membranes are washed to remove excess/unbound antibody with one 15-min rinse followed by four more 5-min rinses using fresh PBS/Tween for each rinse. The choice of enzyme-conjugated secondary antibody depends on the detection technique. Horseradish peroxidase and alkaline phosphatases are the most commonly used secondary antibodies.

Detection

Chemiluminescent assays are very popular due to their high sensitivity and ease of use. Horseradish peroxidase-conjugated secondary antibodies are used for the chemiluminescent systems. We have used the ECL-detection kit (Amersham Life Science, Denver, CO); Supersignal, Supersignal Ultra, and Supersignal Blaze kits(Pierce, Rockford, IL); and Chemiluminescence Reagent Plus (NEN Life Sci, Boston, MA) but at present there are several more companies that have chemiluminescent systems. The three different Pierce kits vary in sensitivity from pico- to femtomoles. All these kits involve a 1:1 mixture of two different reagents that are pipetted onto the protein side of the membrane for 1 min. After 1 min, excess reagent is blotted off by touching the edge of the membrane to a paper towel and then the membrane is wrapped in clear food wrapping paper. The wrapped membrane can then be transferred to an X-ray film-developing cartridge or to a gel doc system. Care should be taken to have the protein side of the membrane facing the film or camera. We use ECL Hyperfilm (Amersham Life Science). Data analysis of the bands can be done using a desktop scanner or a gel doc system to acquire the data from the film and then process the data with computer software that can quantify densiometric data.

Membrane Stripping

Further experiments can also be done with the membranes by stripping them of the antibodies and reblocking and reprobing with another set of antibodies. We have had good success with the following stripping procedure. The blot is washed in 100 ml of 0.1 M citrate/pH 3.5 for 10 min and then rewashed in 100 ml of Tris-buffered saline (TBS) containing 0.5 M Tris/pH 7.5, 0.05 M NaCl, and 0.5% (v/v) IGEPAL (Sigma Chemical Co., St Louis, MO). These two wash steps are repeated two more times. After these wash steps, the membranes are rinsed another two times, 10 min each with TBS containing 0.15 M Tris/pH 7.5 and 0.015 M NaCl. Note the IGEPAL is not used in the final two washes and electrophoresis-purity reagents are used for all other ingredients. The membranes are now ready to be reblocked and reprobed.

TABLE 2

Some Commercial Sources of Various Connexin Antibodies

Connexin type	Source[a]	
	Polyclonal	Monoclonal
Cx30.3	ADI	
Cx31	ADI	
Cx31.1	ADI	
Cx32	ADI, CI, Biogen, Zym	CI, Zym
Cx33	ADI	
Cx37	ADI	
Cx40	ADI	
Cx43	ADI, CI, Zym	CI, TL, Zym
Cx45	ADI, CI	
Cx46	ADI	
Cx50	ADI	

[a] Alpha Diagnostics International, San Antonio, TX (ADI); Biogenesis LTD, Poole, United Kingdom; (Biogen); Chemicon International Inc., Temecula, CA (CI); Tranduction Laboratories, Lexington, KY (TL); Zymed Laboratories, South San Francisco, CA (Zym).

A.11.c. Discussion

Western blot analysis is an excellent way to screen cells for connexins. Currently several companies offer an assortment of primary antibodies to various connexins. Table 2 lists some of the commercially available antibodies. Western blot analysis should complement Northern blot analysis since the RNA message is not always translated. Western blot analysis can also be useful in determining some posttranslational changes. For example, the carboxy terminus of Cx43 has multiple phosphorylation sites that alter the electrophoretic mobility of the connexin. Figure 9 illustrates the three major isoforms of Cx43 typically seen in WB cells, which are normally termed P_0, P_1, and P_2. Pretreating the protein sample with phosphatase results in only the P_0 band, indicating that P_1 and P_2 are two different phosphorylation states of Cx43. Treatment with TPA results in hyperphosphorylation of Cx43 (Fig. 9) where the P_0 band disappears and multiple bands above P_2 appear. Treatment of the protein samples isolated from TPA-treated cells with phosphatase also results in only a P_0 band (data not shown). However, one must be cautious in the interpretation of the data. Although a correlation between GJIC and the change in phosphorylation exists, this does not necessarily indicate a cause and effect. Hyperphosphorylation of Cx43 has been observed to occur in cells with normal GJIC (43).

In conclusion, Western blots offer a sensitive and specific technique to identify various types and forms of

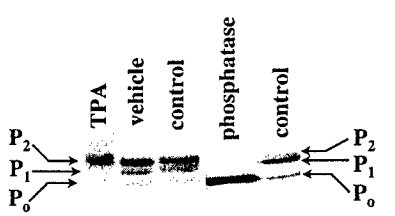


FIG. 9. Western blot analysis of Cx43 from WB-F344 rat liver epithelial cells. WB-F344 cells were treated with TPA (10 ng/ml, 37°C, for 15 min), or vehicle in the absence of TPA (vehicle), or calf intestinal alkaline phosphatase (10 units for 1 h, 37°C). Untreated cells were used as a control. P_0, Unphosphorylated; P_1 and P_2, phosphorylated species of connexin 43.

connexins. In the future, this technique should produce many more fruitful results concerning the biology of gap junctions and the relevance of GJIC to human diseases.

B. Isolation of Human Breast Epithelial Stem Cells

B.1. Background

Stem cells are undifferentiated cells capable of (a) proliferation, (b) self-maintenance, (c) production of a large number of differentiated functional progeny, and (d) regeneration of the tissue after injury and a flexibility in the use of these options (44). In essence, the most basic characteristics of stem cells seem to be the extensive capacity for self-renewal and the ability to generate differentiated daughter cells. Besides these two features, stem cells have been reported to be radiosensitive (45), slow cycling in cell division (46), and contact insensitive (47). Some specific genes have been shown to be expressed in certain stem cells (e.g., $\alpha_2\beta_1$ integrins with epidermal stem cells, α-fetoprotein with liver stem cells, and $CD34^+$ with hematopoietic stem cells). Stem cells may be identified by these markers or other methods such as colony-forming ability, extended time of label retention, and contact-insensitive growth (47, 48). The human breast epithelial cells (HBECs) with stem cell characteristics (Type I HBECs) to be described in this section were discovered by a distinguishable cell and colony morphology associated with these cells that is different from the conventional cell type (Type II HBECs). After characterization, Type I HBECs were found to possess major stem cell features, i.e., the ability to differentiate into other cell types by cyclic AMP-inducing agents and to form ductal and terminal end bud-like structures where mammary stem cells are believed to be located (49, 50).

B.2. Materials

B.2.a. MSU-1 Medium

This medium is a 1:1 mixture (v/v) of a modified Eagle's minimal essential medium (MEM) and a modified MCDB 153 supplemented with human recombinant epidermal growth factor (0.5 ng/ml), insulin (5μg/ml), hydrocortisone (0.5 μg/ml), human transferrin (5 μg/ml), 3,3′,5-triiodo-L-thyronine (1×10^{-8} M) and 17β-estradiol (1×10^{-8} M) (49).

The MSU-1 powder medium without growth factor/hormones and $NaHCO_3$ in 10-liter package prepared by commercial companies is first dissolved in 3 liters Milli-Q-purified water and titrated to pH 6.5 before adding 10.88 g of $NaHCO_3$ and the remaining water and filter-sterilized. The growth factors/hormones are prepared as follows:

Recombinant human EGF (Sigma): Dissolve in PBS as 100 μg/ml stock, then dilute to 10 μg/ml, aliquot in 25 μl/vial, and store in a freezer. Add 25 μl (10 μg/ml) to 500 ml medium.

Insulin (Sigma): Add 19.9 ml sterile H_2O and 0.1 ml glacial acetic acid into a 100-mg bottle; invert and shake to dissolve. Add 0.5 ml (5 mg/ml) to 500 ml medium.

Hydrocortisone (Sigma): Prepare 1 mg/ml in ethanol. Add 0.25 ml (1 mg/ml) to 500 ml medium.

Human transferrin (Sigma): Dissolve 2 mg/ml in PBS and filter-sterilize. Add 1 ml (2 mg/ml) to 500 ml medium.

3,3′,5-triiodo-DL-thyronine (Sigma): Prepare 1 mM (0.65 mg/ml in 0.05 N NaOH) stock, then dilute 100× to 1×10^{-5} M. Add 0.5 ml (1×10^{-5} M) to 500 ml medium.

17β-Estradiol (Sigma): Prepare 1×10^{-4} M stock (1 mg in 18.36 ml ethanol and 18.36 ml PBS). Add 50 μl (1×10^{-4} m) to 500 ml medium.

The MSU-1 medium can support the growth of both Type I and Type II HBECs. However, Type I HBECs may grow faster in MSU-1 medium supplemented with 5% fetal bovine serum (referred to as Type I medium) and Type II HBECs grow better in MSU-1 medium supplemented with 0.4% bovine pituitary extract (referred to as Type II medium).

B.2.b. Collagenase Solution

Collagenase (Sigma, Type 1A) is dissolved in the MSU-1 medium (2.5 mg/ml) and filter-sterilized.

B.3. Establishment of Normal Human Breast Epithelial Cell Culture from Reduction Mammoplasty Tissues

1. Transport tissues in Type I medium using 50-ml centrifuge tubes or other sterile containers on ice.
2. Scrape and remove most of the soft adipose tissues, then mince the remaining tissues into small pieces with a scalpel.
3. Digest tissues in collagenase solution in Erlenmeyer flasks at 37°C in a water bath overnight (1 g tissue in 10 ml collagenase solution).
4. Centrifuge to remove the collagenase solution.
5. Wash the cell pellet with MSU-1 medium and centrifuge to remove the medium.
6. Culture cells in Type I medium in 75- or 150-cm^2 flasks. Allow 2 h for fibroblasts to attach, then transfer cell and cell aggregates in medium to new flasks.
7. After overnight incubation, change to MSU-1 medium.
8. Change medium every 2 days for 1 week (floating tissues or cell aggregates in medium should be collected by centrifugation and replated during medium changes). Store cells in liquid nitrogen in PBS + 10% DMSO.

At Day 5 or 6 after the initiation of culture, fibroblasts may be selectively removed with 5× diluted

trypsin (0.002%) and EDTA (0.02%) from the culture containing both epithelial and fibroblast cells. This procedure works only for the initial culture but not for the subsequent subculture.

Example. Fifty grams of tissue digested in 500 ml collagenase solution overnight was plated in two 150-cm^2 flasks for 2 h for fibroblasts to attach. The floating tissue in Type I medium was transferred to two 150-cm^2 flasks (A) and incubated overnight. The floating tissue in flasks A was centrifuged and transferred to two new 150-cm^2 flasks (B). Both flasks A and B developed Type I and Type II HBECs in MSU-1 medium.

B.4. Starting Normal HBEC Culture from Passage I Cells Stored in Liquid Nitrogen

1. Thaw cells in a vial at 37°C.
2. Add cells in freezing solution (0.8 ml) to a plate with 10 ml Type I medium and inoculate in a 9-cm plate (plate A). Incubate for 2 h in a CO_2 incubator.
3. Transfer unattached cells to a 15-ml tube and centrifuge (1000 rpm, 8 min); add 8 ml Type I medium to plate A.
4. Suspend pelleted cells in 8 ml Type II medium and inoculate in a new plate (plate B); incubate both plates A and B in a CO_2 incubator.
5. After 1 day incubation, shake medium in plate B and immediately transfer the medium containing suspended cells to a 15-ml tube and centrifuge; add 8 ml Type I medium to the pelleted cells, disperse the cells, and transfer to a new plate (plate C). Add 8 ml Type II medium to plate B. Plate B may be washed the second time with agitation using Type I medium to remove loosely attached Type I HBECs (plate C^1).
6. Incubate in a 37°C, 5% CO_2 incubator; renew medium once every 3 days. Plates B and C (C^1) will contain Type II and Type I HBECs respectively, while plate A may be a source for stromal fibroblasts.

B.5. Characterization of Presumptive Human Breast Epithelial Stem Cells

The two types of HBECs have been extensively characterized and were found to differ substantially in phenotype (49, 51, 52). Besides morphological differences, the two types of cells can be separated and grown in pure cultures by two features: differential sensitivity to FBS and early or late attachment on plastic after trypsinization and subculture. Human breast cancers exhibit luminal epithelial cell markers (i.e., epithelial membrane antigen and cytokeratin 18) instead of basal epithelial cell markers (cytokeratin 14 and α_6-integrin) and are predominantly estrogen receptor (ER) positive and deficient in GJIC. These characteristics were shared by Type I but not Type II HBECs. Significantly, Type I HBECs show stem cell characteristics, i.e., the ability to differentiate into other cell types by cyclic AMP inducing agents and to form budding/ductal structures in Matrigel. According to the stem cell theory of carcinogenesis, stem cells give rise to cancer cells by blocking their differentiation and preserve the undifferentiated characteristics of stem cells in cancer cells (53).

TABLE 3

Major Phenotypic Differences between Type I and Type II HBECs

Parameter	Type I	Type II
Cell morphology	Variable in shape	Uniform in shape, cobblestone appearance
Colony morphology	Boundary smooth and restricted	Boundary not smooth
Attachment on plastic surface after trypsinization	Late	Early
Effect of FBS	Growth promotion	Growth inhibition
Gap junctional intercellular communication	Deficient	Efficient
Expression of		
Connexin 26	−	+
Connexin 43	−	+
Epithelial membrane antigen	+	−
Cytokeratin 18	+	−
Cytokeratin 19	+	−
Cytokeratin 14	−	+
α_6-Integrin	−	+
Estrogen receptor	+	−
Effect of cAMP (induced by cholera toxin, forskolin)	Induces Type I cells to change into Type II cells	
Organoid on Matrigel	Budding/ductal structure	Hollow balls
	Acini	Elongated cell mass
Response to SV40 large T antigen		
Anchorage-independent growth	+	−
Spontaneous immortalization	High frequency	Low frequency

We found that Type I HBECs were indeed more susceptible to neoplastic transformation than Type II HBECs. In response to an oncogenic stimulus (SV40 large T antigen), Type I HBECs acquired anchorage-independent growth which was not found for Type II HBECs (6). Furthermore, Type I HBECs were dramatically more susceptible to telomerase activation and immortalization after transformation by SV40 (54). These results support the concept of cancer as oncogeny as blocked or partially blocked ontogeny (14). The major phenotypic differences between Type I and Type II HBECs are listed in Table 3.

DISCUSSION

The hypothesis that the stem cell is a target cell for carcinogenesis was the motivating rationale to try to isolate human stem cells. In our quest to do so, we have isolated two human epithelial pluripotent stem cells [human kidney (47) and human breast (49)]. These two cell types are characterized by their lack of connexin expression and lack of functional GJIC. Additionally, one other presumptive pluripotent stem cell has been reported to lack functional GJIC (55). In addition, the totipotent stem cell, the fertilized egg, lacks GJIC (18). With the majority of evidence showing that most malignant cancer cells, which lack growth control, do not terminally differentiate and readily apoptose and lack functional homologous or heterologous GJIC (3), it seems that a normal stem cell ought to be similar to tumor cells, except for alterations in oncogenes and tumor suppressor genes.

Apparently, the major difference between the two is that the normal stem cell can be readily induced to express its connexin genes and form functional GJIC, whereas the carcinogenic process has rendered GJIC dysfunctional (3). Some cancer cells, such as HeLa, normally have their connexin genes suppressed at the transcription levels (56). However, given the right microenvironmental conditions, a connexin gene can be induced to restore GJIC and growth control (57, 58). Other tumor cells have GJIC disrupted either because of some mutation in their connexin or in some gene that regulates its synthesis, transport, assembly, and function (3).

With the isolation of pluripotent stem cells that appear to be characterized, in part, by the lack of expressed connexins and functional GJIC, it will now be possible to (a) study the mechanism by which the connexin genes are regulated and how the expression of those connexin genes might be related to pluripotent stem cell differentiation; (b) genetically engineer these stem cells for potential therapy of various human heredity diseases; (c) generate cells for tissue replacement of damaged or diseased organs; (d) screen for potential therapeutic or toxic chemicals that could alter the differentiation of stem cells; and (e) study the carcinogenesis of human stem cells when induced to form three-dimensional "organoids."

Finally, with the isolation of human stem cells that are characterized by the lack of connexin gene expression and functional GJIC, it should be possible to determine the controlling mechanisms that give a stem cell the ability to divide symmetrically to expand its numbers or to divide asymmetrically to differentiate yet retain one daughter cell with the ability to maintain stem cell ability. One hypothesis to explain these properties of stem cells seems to be emerging from our studies. These hypotheses can be viewed in Figs. 10A and 10B. In brief, our recent observations have shown that the pluripotent stem cells, when isolated and grown on traditional plasticware, can divide symmetrically for a number of divisions. Yet when these pluripotent stem cells are placed on a more "natural" substrate (i.e., Matrigel), they can form three-dimensional organoids that exhibit the original pluripotent stem cell and their differentiated daughter in a structure that mimics the structure found in the tissue from which it was originally isolated.

This implies that the stem cell–plastic surface interaction sends a signal within the stem cell to divide symmetrically (division plane being vertical) so that both daughters remain attached to the plastic surface. Both daughters receive the same signal as the mother stem cell before division. On the other hand, when the

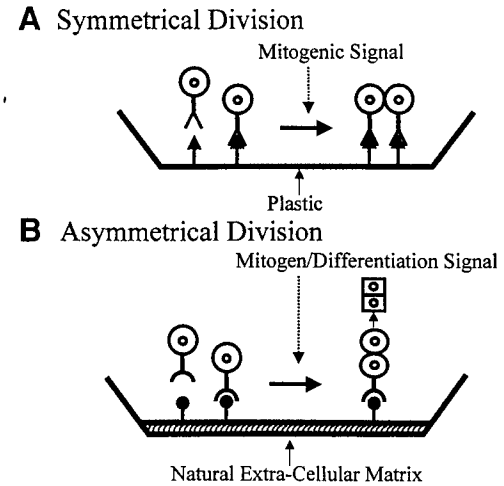


FIG. 10. Diagram illustrating how toti- or pluripotent stems might regulate symmetrical and asymmetrical cell division. (A) Stem cells attached to a particular matrix might receive a cell matrix signal to have its division plane vertical to the substrate giving rise to two cells, both of which are still attached to substrate. (B) The stem cell is attached to another substrate which sends a cell matrix signal to have the cell division plane horizontal to substrate. In this case, one daughter cell remains attached to substrate while the other is free of this signal as there is no GJIC between the daughters.

stem cell attaches to a more "natural" substrate such as collagen, laminin, or fibronectin, the cell divides asymmetrically (the division plane being horizontal to surface) so that one daughter still remains attached to the original surface and the other daughter is detached from that surface. This implies that the one cell still receives the original signal to remain "stem," while the other is now "free" of this restraint. Since there are no expressed gap junctions in the stem cells, there would be no transfer of these substrate–cell signals from one daughter to the sister daughter. This freedom from the gap junction suppression allows the daughter cell on top of its attached, but non-GJIC-coupled sister to express connexin genes and to differentiate.

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